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DISCUSSION ON CURRENT PROBLEMS IN THE BIOCHEMISTRY OF NUCLEIC ACIDS

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1951

FOREWORD

Concerning Supplements to the

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

Despite these considerations, certain authors rightly desire to publish the results of their research in an extensive form at a cost which cannot fairly be assessed against the subscribers; certain observations and data may require such a form of publication. To satisfy these desires and needs the Editorial Board of the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY has approved, on a trial basis, the publication of occasional Supplements to the Journal. The articles published in these Supplements must meet the usual standards of scientific merit; the authors must provide the full cost of publication.

It is hoped that such a Supplement will fulfill the requirements for unique publications and will supply scientific reports of unusual interest to our subscribers at no additional cost to them.

DETLEV W. BRONK

DISCUSSION ON CURRENT PROBLEMS

IN THE

BIOCHEMISTRY OF NUCLEIC ACIDS

GIVEN AT

RESEARCH CONFERENCE FOR BIOLOGY AND MEDICINE OF THE ATOMIC ENERGY COMMISSION

sponsored by

THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY

Oak Ridge, Tennessee
April 13, 14, 1950

OAK RIDGE NATIONAL LABORATORY
Operated by Carbide and Carbon Chemical Company, a Division of Union Carbide and Carbon Corporation for the Atomic Energy Commission

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INTRODUCTION

The importance of nucleic acids to the living cell in general, and with special regard to its response to radiation, has made it desirable to obtain a clearer picture of the present status of the chemistry of these complicated compounds. A discussion of the "Current Problems in the Biochemistry of Nucleic Acids" was the subject of this research meeting held in Oak Ridge in April 1950, and cosponsored by the Biology Division of the Oak Ridge National Laboratory and the Atomic Energy Commission, with the cooperation of the Oak Ridge Institute of Nuclear Studies.

This was the third of the biology conferences held to encourage free and informal discussions in fields of basic importance to the understanding of the effects of radiation on living cells.

The first symposium dealt with radiation genetics, and its proceedings were published as a supplement to this Journal (Supplement I, Vol. 35, June 1950); the second conference, on the relation of radiation to microbiology and some fundamental aspects of radiation, will soon go to press.

The speakers of the Biochemistry of Nucleic Acids Conference were A. M. Michelson, Erwin Chargaff, Gerhard Schmidt, Berwind P. Kaufmann, Arthur W. Pollister, George Bosworth Brown, John M. Buchanan, Dwight B. McNair Scott, William Shive, Helen R. Skeggs, Herbert S. Loring and Alfred E. Mirsky.

Two of the papers were not submitted by the authors for inclusion in this monograph. The paper by Waldo E. Cohn was included since it covers an important phase of nucleic acid chemistry which was brought up many times in the discussions. Dr. Charles E. Carter and Dr. Waldo E. Cohn were largely responsible for the selection of speakers for this program.

It is fitting that acknowledgment be made here of the contribution of Dr. W. E. Cohn, Dr. D. G. Doherty, Dr. Elliot Volkin and Dr. S. F. Carson, of this Laboratory, in reviewing these assembled papers and in serving as advisors to the editorial staff.

ALEXANDER HOLLAENDER

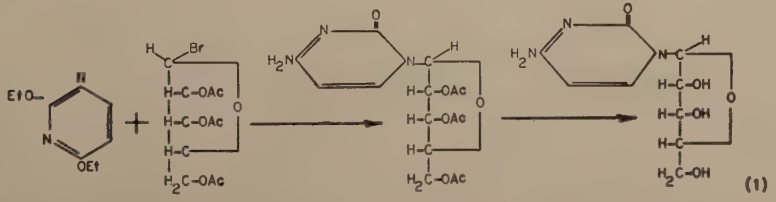
February 1951

ORGANIC CHEMISTRY OF NUCLEIC ACIDS

A. M. MICHELSON
California Institute of Technology, Pasadena

In the synthesis of nucleosides, 4 methods have emerged: (a) condensation of the acetohalogen sugar with 2,6-dialkoxypyrimidines (Hilbert and Johnson, '30), (b) interaction of an acetohalogen sugar with the silver salt of the pyrimidine or purine (Fischer, '14), (c) condensation of sugars with certain 4,6-diaminopyrimidines, followed by synthesis of the glyoxaline ring (Todd et al., '43 to '50), (d) ring closure of a suitable glyoxaline glycoside derivative (Baxter and Spring, '47).

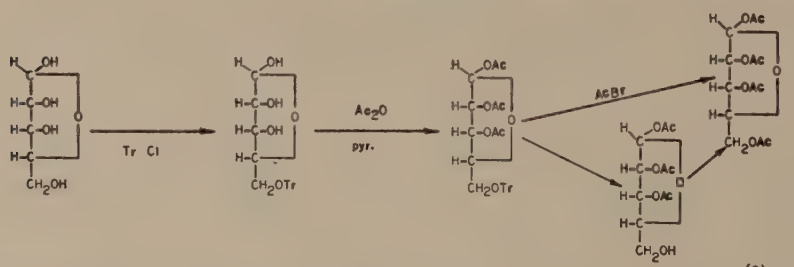
Cytidine (3-β-D-ribofuranosidocytosine) identical with the natural nucleoside, has been synthesized by the first method (Howard, Lythgoe, and Todd, '47). Interaction of 2,6-diethoxypyrimidine with acetobromoribofuranose gave a gummy product which on treatment with methanolic ammonia yielded a mixture of bases, from which pure cytidine sulphate was obtained after fractionation of the picrates. This method has been used by Visser and co-workers ('48) to prepare a number of pyrimidine glycosides.



The furanose sugar derivative used in these investigations was prepared by treatment of 1,2,3,5-tetracetyl-D-ribofuranose

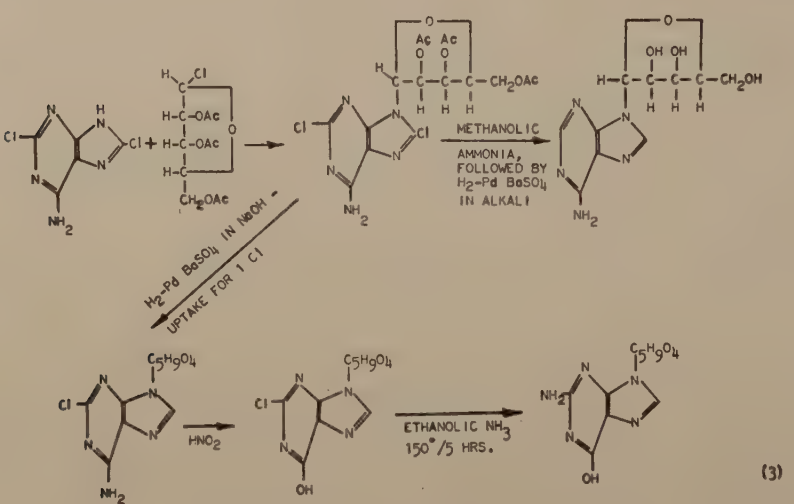
with liquid hydrogen bromide, according to the method of Schlubach and Wagenitz ('32).

Tetracetyl-D-ribofuranose itself is readily obtained from 1,2,3-triacetyl-5-trityl-D-ribose by removal of the trityl group by hydrogenolysis and acetylation (Howard, Lythgoe, and Todd, '47) or directly by treatment with acetyl bromide



(2)

(Bredereck and Hoepfner, '48). Both adenosine and guanosine have been synthesized by the second method, as shown in the series of reactions outlined below (Davoll, Lythgoe, and Todd, '48).

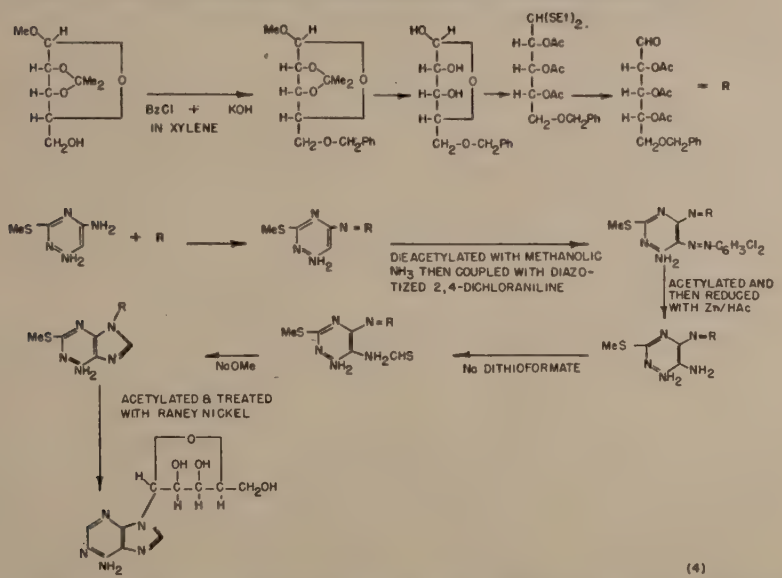


(3)

Acetochlororibofuranose (prepared from the tetracetyl-ribose by treatment with ethereal hydrogen chloride) was refluxed with the silver salt of 2,8-dichloroadenine in xylene,

to give the triacetyl-2,8-dichloroadenine riboside. Complete removal of halogen from the deacetylated glycoside by hydrogenation yielded adenosine, while removal of the more active 8-chloro atom only, followed by deamination of the 6-amino group with nitrous acid and amination of the 2-chloro with ethanolic ammonia gave guanosine, identical in all respects with the natural product.

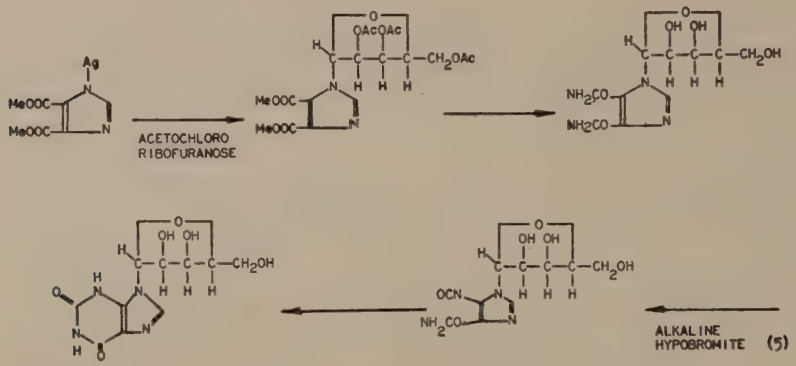
Cyclization of a glyoxaline ring onto a 6-amino-4-ribofuranosidopyrimidine to yield adenosine (Kenner, Taylor, and Todd, '49) affords an unambiguous proof of the structure of this important nucleoside. The synthetic route established in this work is indicated schematically in equation 4.



2,3,4-Triacetyl-5-benzyl-D-ribose was prepared from D-ribose and condensed with 4,6-diamino-2-methylthiopyrimidine to give the Schiff base, which after deacetylation was coupled with diazotized 2,5-dichloroaniline. The crude azo compound was acetylated, purified by chromatography and reduced with zinc dust and acetic acid to the 5-amino compound, which was directly thioformylated. The thioformamidoglycoside was then cyclized with sodium methoxide, acetylated and heated with

Raney nickel in ethanol to remove both the benzyl and methylthio groups. Deacetylation of the product gave adenosine (9-β-D-ribofuranosidoadenine) identical with that obtained from natural sources.

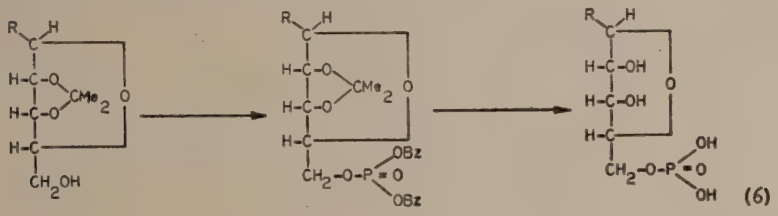
The synthesis of xanthosine (Howard, McLean, Newbold, Spring, and Todd, '49) by building up the pyrimidine moiety on a glyoxaline glycoside, is an example of the 4th method for the synthesis of nucleosides. Acetochloro-D-ribofuranose was condensed with the silver salt of methyl glyoxaline 4,5-dicarboxylate to yield a product, which on treatment with ammonia gave 1-D-ribofuranosidoglyoxaline-4,5-dicarboxyamide. By application of the Hofmann reaction to this compound, xanthosine was obtained.



Phosphorylation of nucleosides to the corresponding nucleotides was, until recent years, largely unsuccessful due to the limitations of the phosphorylating agents used. Owing to the sensitivity of the compounds being phosphorylated, of necessity mild conditions must be employed, thus eliminating many of the earlier known methods for phosphorylation of alcohols such as direct phosphorylation with phosphoric acid, the action of phosphoric anhydride, use of ethyl metaphosphate, which in addition to requiring conditions which would degrade the glycoside, involve some uncertainty as to the actual nature of the product, generally a complex mixture.

With the advent of dibenzyl chlorophosphonate as a phosphorylating agent, however, (Atherton, Openshaw, and Todd,

'45), a number of nucleotides have been prepared, including ADP and ATP (Baddiley and Todd, '47; Baddiley, Michelson, and Todd, '49; Michelson and Todd, '49b). General application of the reagent to a number of protected nucleosides showed that yields were good and that the benzyl groups could invariably be readily removed by hydrogenation, e.g., phosphorylation of 2′,3′-isopropylidene adenosine, uridine, and cytidine and of 2′,3′-isopropylidene methyl-D-ribofuranoside gave good yields of the corresponding 5′-phosphates, after removal of the protecting groups. This reagent is also of importance in that one benzyl group can readily be removed from a d benzyl nucleotide, offering possibilities of polynucleotide synthesis.



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INVESTIGATIONS ON THE PROBLEM OF THE RIBONUCLEOSIDE-2′-PHOSPHATES

D. M. BROWN, L. J. HAYNES AND A. R. TODD
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(Read by Dr. Michelson)

In the work directed toward the unambiguous synthesis of the mononucleotides derived from the natural ribonucleosides, the preparation of a compound believed to be adenosine-2′-phosphate was described (Michelson and Todd, '49a). Carter and Cohn ('49) had isolated from a yeast nucleic acid hydrolysate two isomeric adenylic acids, neither of which was identical with adenosine-5′-phosphate (muscle adenylic acid). It was suggested, on good evidence, that these compounds, termed adenylic acid *a* and adenylic acid *b*, were respectively the hitherto unknown 2′-phosphate and the 3′-phosphate of adenosine.

The synthetic adenosine-2′-phosphate, originally described as amorphous has now been obtained in crystalline form and direct com-

parison of the x-ray powder photographs of this material with those of samples of adenylic acids *a* and *b*, kindly supplied by Drs. Carter and Cohn, showed that the three compounds were different. Adenylic acid *b* gave an x-ray powder diagram identical with that of adenosine-3′-phosphate. The synthetic "2′-phosphate," however, had an x-ray powder diagram identical with that of adenosine-5′-phosphate (muscle adenylic acid). Furthermore, comparison by this method of the synthetic compound described in the literature as cytidine-2′-phosphate with cytidine-5′-phosphate showed that these two compounds also were indistinguishable from each other.

Accordingly, a further examination was made of the synthetic 2′-phosphates prepared in these laboratories. Titration of adenosine-2′-phosphate and cytidine-2′-phosphate with sodium metaperiodate (Lythgoe and Todd, '44) showed that each consumed approximately one mole oxidant per mole; this demonstration of the presence of an *a*-glycol structure confirmed the conclusion that these two compounds are 5′-phosphates. We subjected to periodate oxidation original specimens of the compounds described in the literature as uridine-2′-phosphate and cytidine-2′-phosphate (Gulland and Smith, '47, '48), which were kindly supplied by Dr. H. Smith as the dibrucine and barium salts respectively; both materials absorbed one mole periodate per mole and they must therefore be 5′-phosphates. The adenylic acid *a* isolated by Carter and Cohn was unaffected by the periodate reagent. This, together with its distinction from both adenosine-3′-phosphate and adenosine-5′-phosphate is good evidence for its formulation as a 2′-phosphate.

The synthetic compounds previously considered to be 2′-phosphates had been prepared by treatment of the benzylidene derivatives of adenosine, guanosine, and cytidine with dibenzyl chlorophosphonate and subsequent removal of the protecting groups by hydrogenolysis and dilute acid treatment (Michelson and Todd, '49a), or by an analogous method using diphenyl chlorophosphonate as phosphorylating agent (Gulland and Smith, '47, '48). That this was a valid route to 2′-phosphates seemed warranted on the evidence of Bredereck and Berger ('40) and of Gulland and Overend ('48) that the condensation product of benzaldehyde and guanosine was 3′,5′-benzylidene-guanosine. Bredereck and Berger were unable to prepare a triphenylmethyl derivative and also stated that the compound did not give the Klimek-Parnas test. Gulland and Overend acetylated benzylidene guanosine, removed the benzylidene group by mild acid hydrolysis and found that the acetyl guanosine so obtained was not oxidized by periodate. It was therefore considered by them to be 2′-acetyl guanosine. Further, by methylation of acetyl benzylidene

guanosine with dimethyl sulphate and alkali followed by hydrolysis, isolation of a methylribose, and hydrogenation of this to an optically active methylribitol, Gulland and Overend ('48) demonstrated the absence of a free 3′-hydroxyl group in benzylidene guanosine.

On the basis of this evidence, and assuming the benzylidene derivatives of all 4 nucleosides to be analogously constituted, the origin of 5′-phosphates in the synthetic experiments above mentioned is obscure, and we were led to consider, at this stage, the possibility of phosphate migration during the synthesis; evidence was sought on this point (Brown, Haynes, and Todd, '50).

No evidence of rearrangement was found when the natural adenylic acid *a* (adenosine-2′-phosphate) of Carter and Cohn was treated with acid under the conditions used to remove the benzylidene group in the synthetic experiments. It may be pointed out, incidentally, that no 5′-phosphates have as yet been isolated from pentose nucleic acid hydrolysates. Dilute acetic acid treatment of benzylidene adenosine dibenzyl phosphate gave a high yield of adenosine-5′-benzyl phosphate, identified by direct comparison with an authentic specimen (Baddiley and Todd, '47) by x-ray powder photographs and by periodate titration. The migration of a monobenzylphosphoryl group in this case and of a monophenylphosphoryl group in the experiments of Gulland and Smith ('47, 48), which would be required to explain these results, seemed rather unlikely, and a reinvestigation of the structure of the benzylidene nucleosides was clearly necessary.

Periodate titration of the acetyl adenosine obtained by Michelson and Todd ('49a, b) by acetylation of benzylidene adenosine followed by removal of the benzylidene group showed an uptake of one mole oxidant per mole. Repetition of this preparation under conditions designed to preclude a possible acetyl migration under alkaline conditions (Helferich and Klein, '26, '27) gave the same acetyl adenosine which we have now also prepared from 2′,3′-isopropylidene adenosine. This compound must now be regarded as 5′-acetyl adenosine.

When benzylidene guanosine, having the same melting point and other characteristics as that prepared by Gulland and Overend ('48), was acetylated by the method described by these authors, we obtained only amorphous products. Acetylation with acetic anhydride in pyridine, however, yielded a crystalline monoacetate from which we were able to remove the benzylidene group by treatment with 30% acetic acid at 70°C. The crystalline acetyl guanosine (melting point 192–193°C.) so obtained did not correspond closely in melting point to the acetyl derivative (melting point 176–178°C.) reported by Gulland and Overend. We found that our compound consumed one mole metaperiodate per mole and hence it is clearly 5′-acetyl guanosine.

The source of this discrepancy between our findings and those of Gulland and Overend is not clear, but their formulation of benzylidene guanosine as a 3′,5′-derivative is not valid in the light of our experiments. In fact our observations leave very little doubt as to the 2′,3′-structure of all 4 benzylidene nucleosides. We are engaged in the characterization of the methylribose derived from the methylation of acetyl benzylidene guanosine to furnish final proof of the structure of this compound. Meanwhile, A. Anderson of Nottingham University (private communication) has provided support for our contention by the observation that Gulland and Overend's alleged 2-methylribose could not be distinguished from authentic 5-methylribose on paper chromatograms.

Experiments are at present in progress to prepare the 2′-phosphates of the nucleosides by synthesis. Michelson and Todd ('49a, b) reported that the phosphorylation of ditrityl adenosine and 5′-trityl uridine appeared to yield mainly the corresponding 3′-phosphates since, on working up, these compounds were isolated. Further experiments have, however, shown that the 2′-phosphates are also produced in this reaction and that phosphorylation of 5′-acetyl adenosine yields a mixture of the 2′- and 3′-phosphates, containing substantial amounts of the former.

Since the evidence now available indicates that the benzylidene group in the benzylidene nucleosides occupies the 2′,3′ position, it follows that none of the syntheses recorded in the literature for nucleoside-3′-phosphates is unambiguous. This is of some importance in so far as there is as yet no clear proof that the phosphate residue in the natural pyrimidine nucleotides occupies the 3′ position rather than the 2′ position; we hope that further work will settle this point.

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SOME RESULTS OF THE APPLICATIONS OF ION-EXCHANGE CHROMATOGRAPHY TO NUCLEIC ACID CHEMISTRY [1]

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Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

SIXTEEN FIGURES

Ion-exchange (Kunin, '49; Nachod, '49), while used on an industrial scale for many years for water-softening, came into its own as a chemical separation method during the period of 1943 to 1946 at what is now the Oak Ridge National Laboratory. Ion-exchange column chromatography there made it possible to separate precisely the very small but highly radioactive masses of fission products, including those which are rare earths, and the method was soon shown to be the first means for quantitative separation of these notoriously inseparable elements (Tompkins, Khym, and Cohn, 47; Harris and Tompkins, '47; Ketelle and Boyd, '47; Tompkins, '49; Cohn et al., '48).

In a certain sense, the degradation products of nucleic acids — the purine and pyrimidine bases, their ribosides and desoxyribosides and the phosphate esters of the latter — can be considered as analogous to the rare earths in that they form mixtures which are sometimes very difficult to resolve for analytical or preparative purposes (Levene, '31). Accordingly, a deliberate attempt was made to apply the same technique which had proved so successful in the inorganic field to the separation of these substances. While paper chromatography, another new separation device of

[1] Work performed under Contract W-7405-eng-26 for the Atomic Energy Commission.

high resolving power, has recently almost eliminated this analytical problem (Vischer and Chargaff, '48; Chargaff et al., '49; Carter, '50; Chargaff, '50), there were, at least in 1947, many aspects to which ion exchange might contribute and several in which it remains somewhat unique. Among the latter is included its ability, which first dramatically illustrated the power of the method, to resolve isomers of all 4 of the ribonucleotides. This, together with the separation of 5 desoxynucleotides and of the adenosine polyphosphates, will be presented in what follows.

PRINCIPLES OF THE METHOD

The substances of interest to us here are, in contradistinction to the inorganic ions of the earlier work, amphoteric electrolytes whose charge depends, both quantitatively and qualitatively, upon the pH of the medium. Since the operational idea is to absorb at the top of a column (see fig. 1) under one set of conditions and to elute at another, one can take advantage of the acidic and basic dissociation constants of the nucleic acid degradation products in the same way that the citrate and tartrate complex dissociation constants of polyvalent cations were utilized to effect the earlier inorganic cationic separations. Regulation of pH in the latter case afforded control of the distribution coefficient by controlling the degree of complexing and hence the net charge per ion. In the case of the organic acids and bases, pH affects directly the net charge per ion and hence, to a large degree, the distribution coefficient.

The variation of calculated net charge per ion with the pH of the medium for several nucleotides is shown in figure 2 (Cohn, '50a); the pK values used to find the extent of ionization of the various ionizable groups (phosphate and amino) were taken from Levene and Bass ('31). From these curves, one would expect a strong affinity of all nucleotides for anion exchangers in the region of pH 6 and up, where the phosphate group becomes doubly charged and the cationic groups have lost their positive charge, with less and less affinity (lower

distribution coefficient) as the pH falls. Conversely, in the low pH region, one would expect all the nucleotides with amino groups to adsorb on cation exchangers. In both cases, elution is accomplished by either (1) increasing concentration of competing ion (e.g., Cl^- in the case of anion exchange), or

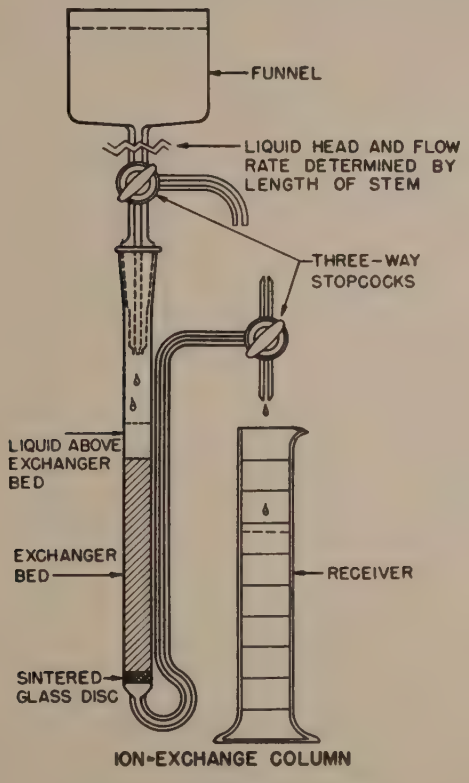

Fig. 1 Ion-exchange column.

(2) changing the pH in the direction of lowering or reversing the effective net charge.

In general, the second method, elution by lowering net charge at minimum ionic strength, is preferable as the substance is recovered in a solution of low ion content which is suitable for concentration by evaporation or by reabsorptior

on a smaller ion-exchange column. However, there are some cases (e.g., the separation of uridylic acid isomers, Cohn, '50b) in which separation is achieved only in pH regions where the net charge is high, thus requiring a medium of higher ionic strength than would be necessary at lower net charge to have the same distribution coefficient.

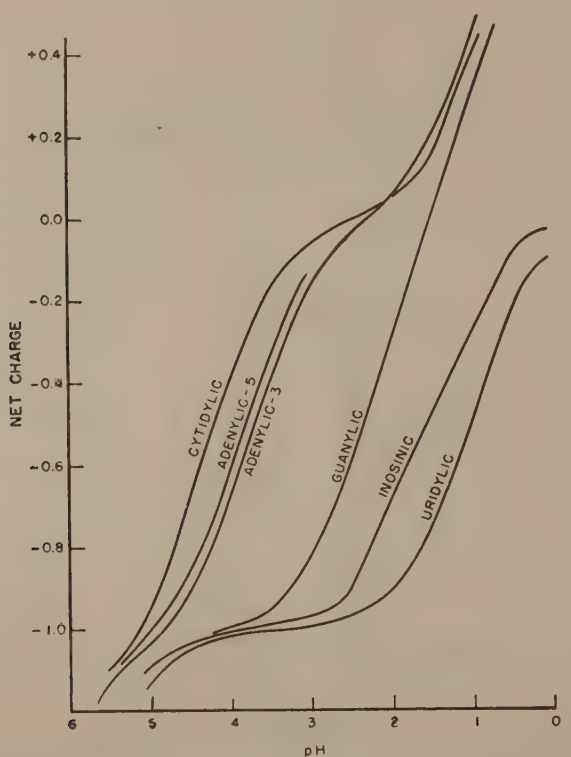

Fig. 2 Net charge per molecule of ribonucleotide as a function of pH (calculated from data given by Levene).

APPLICATION TO SYNTHETIC MIXTURES

While all the factors governing the affinity of such complex solutes for an ion exchanger are not available, it is known that net charge is not the only one. Hence it is not surprising to note that the order of elution of nucleotides from either

cation exchangers or anion exchangers is not always in the exact order predicted from figure 2. In figure 3 (Cohn, '49) is shown a separation based on cation exchange and utilizing elution by hydrogen ion; cytidylic acid precedes adenylic acid, which is not the predicted order. Figure 4 (Cohn, '50a) demonstrates a separation basesd upon anion exchange and utilizing elution by charge reversal; uridylic precedes guanylic

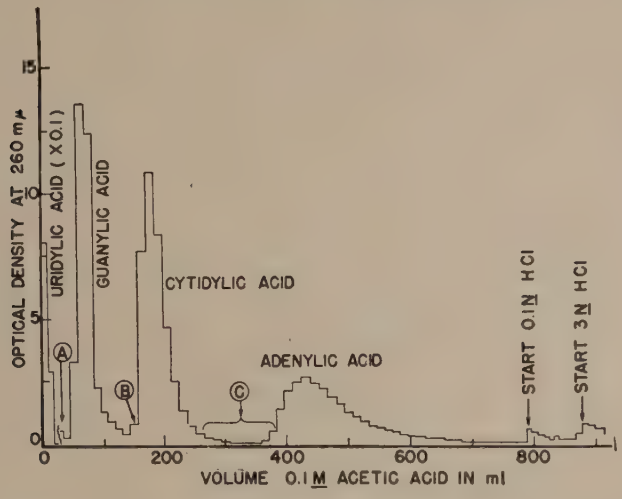

Fig. 3 Separation of mononucleotides from yeast nucleic acid by a cation-exchange column.

Exchanger: Dowex-50, ~ 300 mesh, H^+ form, 24 cm × 0.74 cm².

Eluting Solution: 0.1 *M* acetic acid, 0.15 ml/min.

Test Material: ~ 100 mg mixed nucleotides from $Ba(OH)_2$ hydrolysis of yeast nucleic acid (composition unknown) in 0.1 *M* HAc.

Recoveries: 75–100% (based on optical density at 260 mμ).

Cross-contaminated Material: 0.25% at (A), 0.4% at (B), 1.0% at (C).

acid, which is not the predicted order. Experiments covering a range of pH values indicate that the relative positions of the pyrimidine nucleotide peaks are in agreement with net charges of 37% of the calculated values with respect to the purine nucleotides; the data is summarized in figure 5 (Cohn, '50a). The reason for this difference between purine and pyrimidine compounds is not known but the difference clearly

indicates that structural characteristics may somewhat modify the net charge criterion.

The shape of the elution curve (using purified substances to form an artificial mixture) is shown in figure 4. Upon the observed elution curve (the histogram) are superimposed theoretical curves calculated according to the theory of Mayer

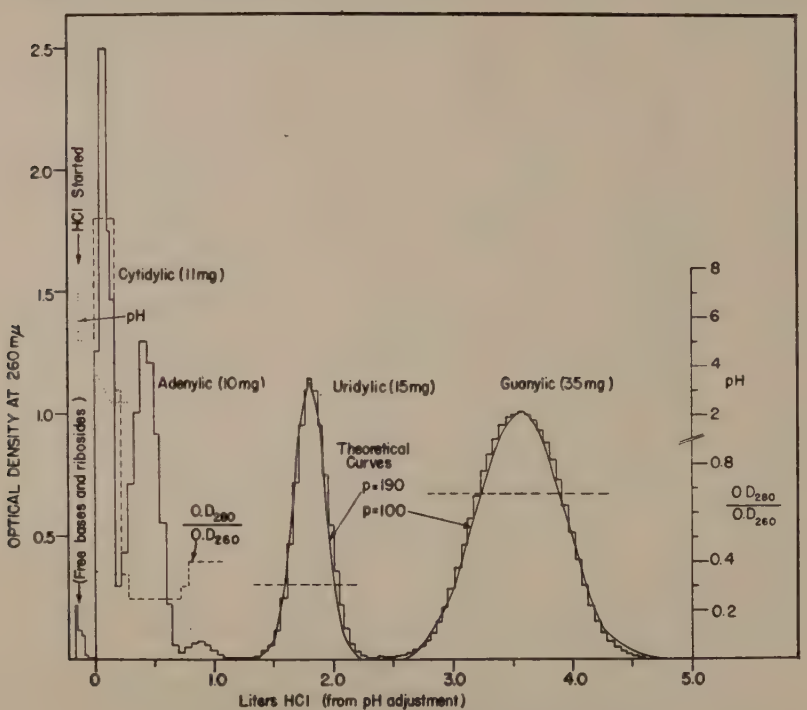

Fig. 4 Separation of mixed monoribonucleotides. Exchanger: Dowex-2 (Cl^-), 0.94 cm^2 × 12.5 cm. Solution: 0.003 *N* HCl, 0.8 ml/min.

and Tompkins ('47), which predicts a symmetrical distribution curve if equilibrium is attained in the column.

Also in figure 4 is shown how the progress of elution is followed with the Beckmann ultraviolet spectrophotometer, each peak being identified by characteristic spectral absorption ratios.

Bases and ribosides do not possess the acidic phosphate group of the nucleotides. However, all, with the exception of adenosine, cytosine and cytidine, have acidic pK's in the range of 8–12 and can therefore be absorbed readily on anion exchangers. One separation of these by elution with buffers in this pH range, chloride being the competing ion, is shown in figure 6 (Cohn, '49). The ribosides (not shown) tend to overlap the corresponding bases. Adenosine is an exception;

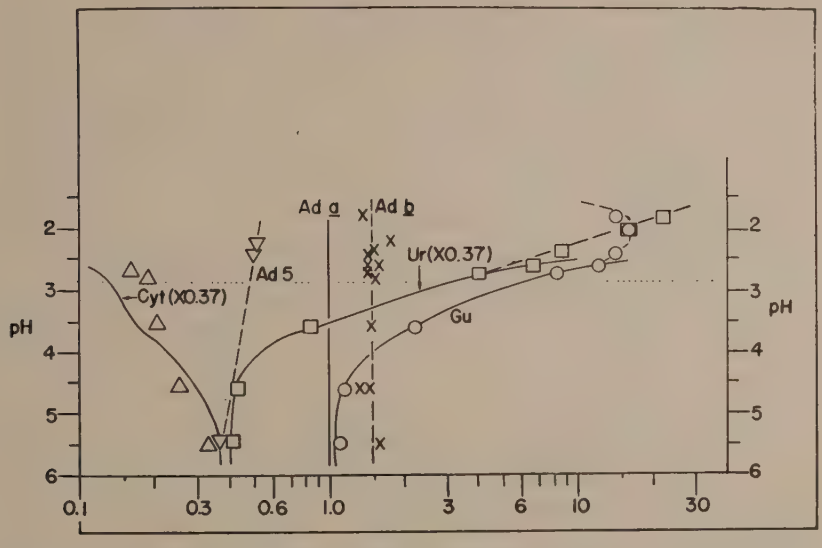

Fig. 5 Observed relative distribution coefficients (points, broken lines), and calculated relative negative charges (solid lines) of nucleotides as functions of pH.

the loss of the hydrogen atom from the 9-position of adenine results in the loss of most of adenine's acidic properties, making adenosine difficult to separate from the cytosine compounds but easily separable from adenine (Cohn, '50a). Guanosine is also separable from guanine (Cohn, '50a) as is xanthosine from xanthine, but inosine and hypoxanthine do not separate appreciably from each other in this system.

Cation-exchange separations, based upon elution by raised concentration of competing ion, are shown in figure 7 (Cohn,

'49). Thymine is inseparable from uracil in this system, as are thymidine and uridine; these are not cations and are not adsorbed. Cytidine precedes cytosine (as do xanthine and hypoxanthine) while 5-methylcytosine follows it closely. The nucleotides are, in contrast to the above, very weakly absorbed on cation exchangers, although it is possible to separate

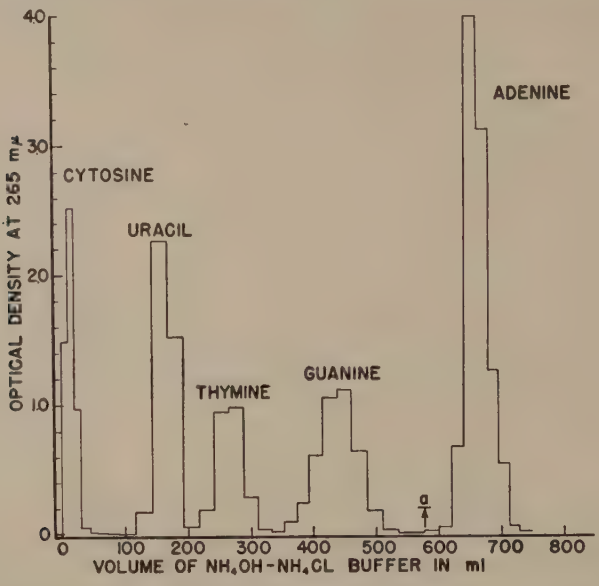

Fig. 6 Separation of purine and pyrimidine bases by an anion-exchange column.

Exchanger: Dowex-1, ~ 300 mesh, Cl^- form, 85 mm × 0.74 cm^2.

Test Material: 1 mg cytosine, 2 mg each of other bases in eluting solution, 0.25 ml/min.

Eluting solution: 0.2 *M* NH_4OH — NH_4Cl buffer, pH 10.6, 0.025 *M* Cl^- (at a, pH → 10.0, Cl^- → 0.1 *M*).

Recoveries: 97.5–99% (based on 265-mμ densities).

them this way (fig. 3). Those compounds which are prone to acid hydrolysis (e.g., purine ribosides and nucleotides) exhibit a significant degree of destruction during their passage through a column of this type. Hence, for these, anion exchange is to be recommended. The use of buffers in conjunction with cation exchange, so successfully applied to

amino acid separations by Stein and Moore ('50), has not been investigated because of its limited usefulness for the separation of these compounds.

APPLICATION TO NATURAL MIXTURES

The first application of the methods described above was to the separation of the products of alkaline hydrolysis of ribonucleic acids isolated from various sources. Figure 8

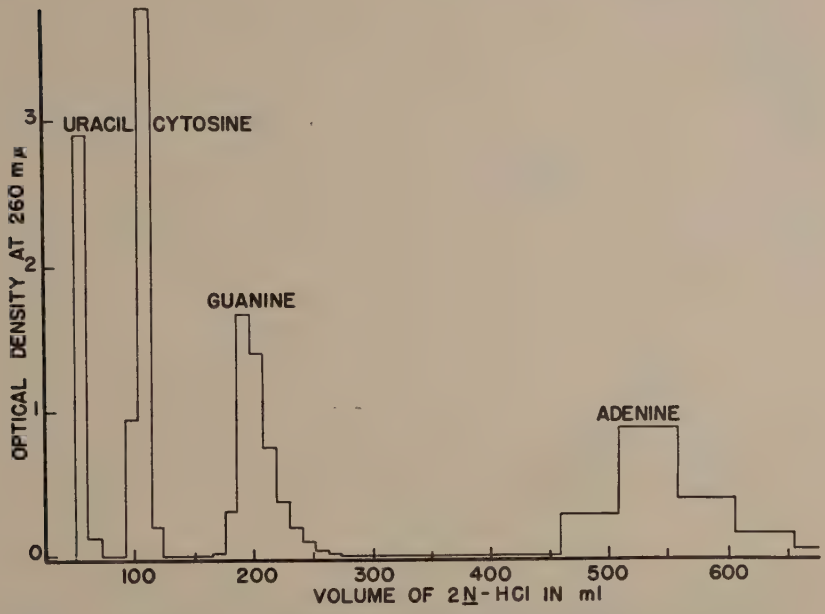

Fig. 7 Separation of purine and pyrimidine bases by a cation-exchange column.
Resin: Dowex-50, ~ 300 mesh, H^+ form, 81 mm × 0.74 cm².
Eluting solution: 2 *N* HCl, 0.6 ml/min.
Test material: 0.5 mg uracil, 1.0 mg each of cytosine, guanine, adenine, in 7.5 ml 2 *N* HCl.
Recoveries: 98.5–101.5% (based on optical density at 260 mμ).

is the elution diagram of such a mixture (Cohn, '50a) and indicates the presence of two unexpected peaks, one in the adenylic acid section and one in the guanylic section of the chromatogram. That the extra peaks represented new com-

pounds seemed obvious from the symmetry of single-component curves such as appear in figure 4. Isomerism was initially postulated because of the close relationship of the new peaks and their identical spectrophotometric properties. This was further supported by showing that the bases and ribosides derived from each were identical. Subsequently, it

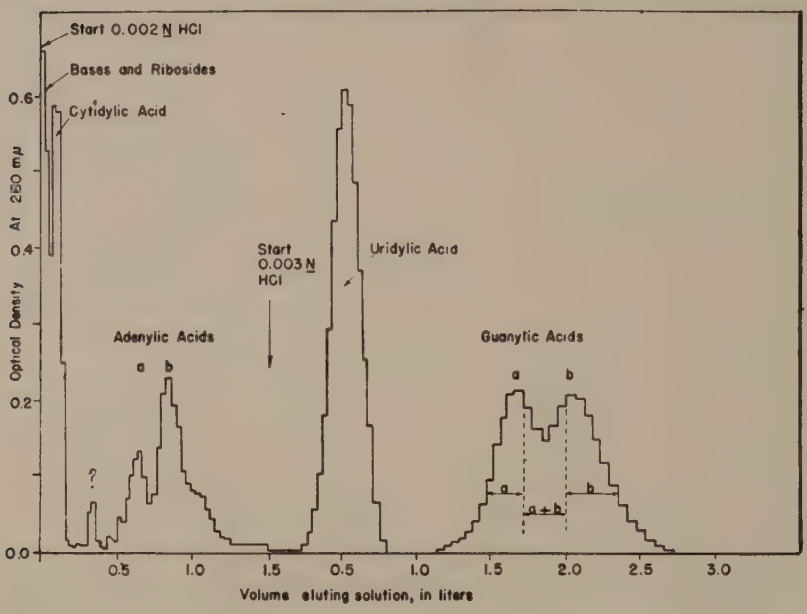

Fig. 8 Separation of mononucleotides of rat liver ribonucleic acid by anion exchange.

Exchanger: Dowex-1, 12.5 cm × 0.74 cm².

Eluting solution: Dilute HCl, 0.5 ml/min.

Test material: ~ 14 mg mixed nucleotides from $Ba(OH)_2$ hydrolysis of rat liver nucleic acid, in 10 ml H_2O.

was shown that acid catalyzes the conversion of either member of any pair into an equilibrium mixture of both.

The first experiments to ascertain the nature of the isomerism indicated that neither of the two adenylic acids (the first nucleotide to show isomerism) was the well-known muscle adenylic acid (adenosine-5′-phosphate) (see fig. 9) and that

the isomerism was independent of the amino group since three inosinic acids could be isolated from the partial deamination of a mixture of the three adenylic acids (fig. 10).

Subsequently, isomerism in cytidylic and uridylic acids was demonstrated (fig. 11) (Cohn, '50b). In the latter substance, this required, as in the case of the inosinic acids (fig.

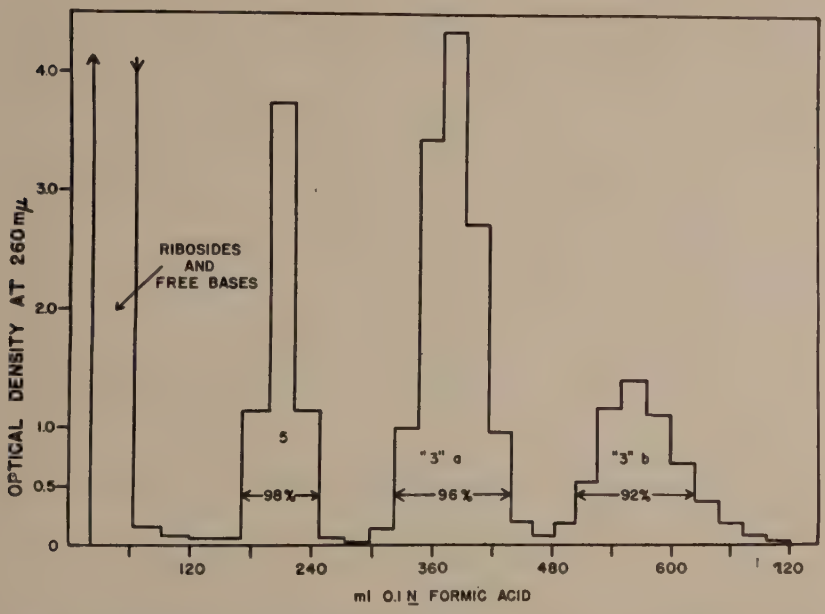

Fig. 9 Separation of adenosine-5-phosphate and the two adenylic acids of yeast nucleic acid by anion exchange.

Exchanger: Dowex-1, 250–500 mesh, 14.0 cm × 0.74 cm².

Eluting solution: 0.1 *M* formic acid (pH 2.4), 0.5 ml/min.

Test material: Commercial adenylic acids, ~ 20 mg total.

10), elution at higher pH with compensatory higher ionic strength. The acid stability of the pyrimidine nucleotides lent itself to the demonstration of ready interconvertibility of the isomers (30 minutes, 0.1 *N* hydrochloric acid, 100°C.) without the large degree of hydrolysis observed in the purine compounds.

Returning to the adenylic acids (designated *a* and *b* as in figs. 8 and 9), it was desired to produce these in gram quantities for chemical and enzymatic characterization. An ion-exchange column of larger cross-sectional area but similar height was set up and the operation of figure 8 was repeated as shown in figure 12. Each fraction was made alkaline with

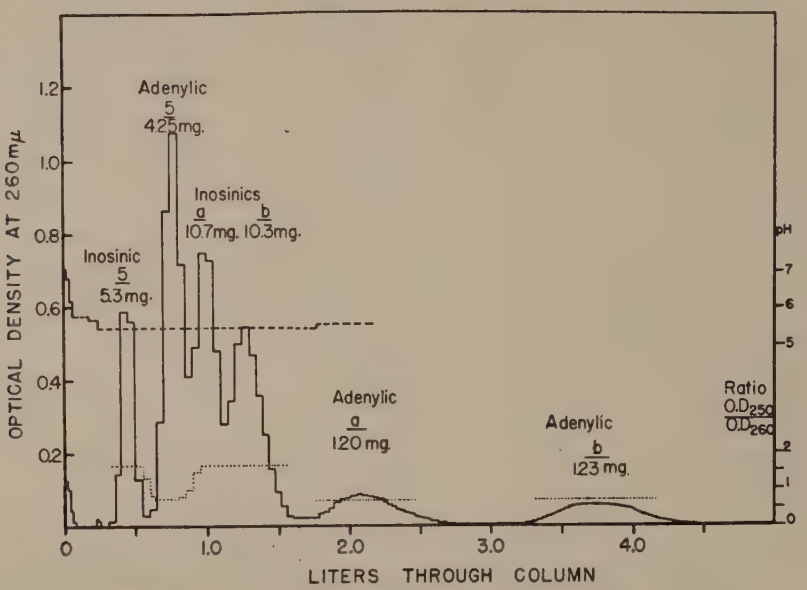

Fig. 10 Separation of three adenylic acids and the corresponding inosinic acids by anion exchange.

Exchanger: Dowex-1 (Cl), 250–500 mesh, 9.5 cm × 0.9 cm².

Eluting solution: 0.02 *M* NaCl, 0.01 *M* acetate buffer, pH 5.5, 1.15 ml/min.

Test material: Commercial adenosine phosphates (3 and 5) and inosinic acids derived from them by nitrous acid deamination.

Recoveries: 96% (based on optical density at 260 mμ). Identification made by ratio of optical density at 250 mμ to that at 260 mμ (dotted line).

ammonia and put through a smaller column (about 8 cm² × 2 cm in height) to reabsorb the adenylic acid. This column was then eluted with 0.01 *N* hydrochloric acid + 0.1 *N* sodium chloride, resulting in adenylic acid solutions of 10–20 mg/ml, from which much of the acid crystallized on standing in the cold, perhaps with the aid of a little alcohol.

The separated isomers were studied with the results shown in table 1. The difference in solubility immediately indicated the possibility of preparing the pure isomers by fractional crystallization. Furthermore, the isomer more insoluble in water (*b*) has properties identical with those given by Levene ('31) for yeast adenylic acid, adenosine-3′-phosphate, which is consistent with Levene's careful recrystallization of his preparations. The new isomer, the structure of which is yet to be established, is that designated as *a*. It may be inferred

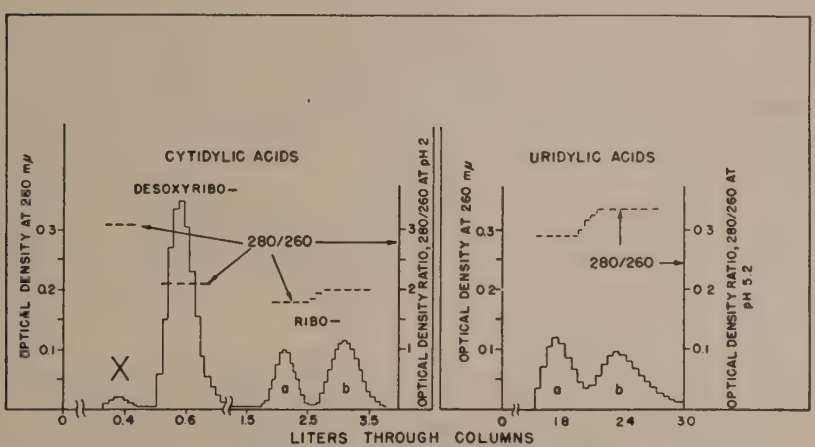

Fig. 11 Separation of cytidylic acids and uridylic acids by ion exchange.
Exchanger: Dowex-1, 200–400 mesh, 11.3 cm × 0.86 cm² (cyt.), 9.5 cm × 0.76 cm² (ur.).
Eluting solution: 0.025 *M* HAc, 0.025 *M* NaAc (cyt.). 0.04 *M* Na formate, 0.0004 *M* formic acid, pH 5.2 (ur.).
Test material: Nucleotides isolated by ion exchange from nucleic-acid degradations.

that this holds true for the other nucleotide isomers also as the commercial products show a higher *b* content the more pure they are found to be; samples of commercially available uridylic and cytidylic acids have been found to be pure *b*.

Although no thorough search has been made, there is at least one enzyme preparation which has been found to discriminate between the two isomers. A relatively crude extract

of potato will split off the phosphate group of both forms at pH 6 but only that of *b* at pH 8 (see table 1). Such differences imply biological significance to the isomerism which is strengthened by the finding by Kornberg ('50) that the adenosinemonophosphate isolated from triphosphopyridinenucleotide seems to be the *a* isomer.

Although the postulate that the new isomers are 2′-phosphates is most tempting, no evidence in favor of this hypothesis has yet been obtained and synthetic material is not yet available for comparison (Brown, Haynes, and Todd, '50).

TABLE 1

Some properties of the ribose adenylic acid isomers

	a	b	5′
Solubility in water	ca. 4 mg/ml	ca. 1 mg/ml	
m.p.	176–8	195–8	
P hydrolysis in acid [a]	65%	65%	2%
pK′ of second phosphate [b]	6.21	6.06	6.48
αD^{20} in alkali	— 62	— 55	
Phosphatase hydrolysis, pH 8 [c]	—	+	+
Phosphatase hydrolysis, pH 6 [c]	+	+	+
Muscle adenylic deaminase hydrolysis	—	—	+
Copper complex formation [d]	—	—	+

[a] 1 *N* HCl, 100°C., 30 minutes; determined by C. E. Carter.
[b] Determined with hydrogen electrode by M. Kuna.
[c] Determined with potato phosphatase by C. E. Carter.
[d] Method of Berlin and Westerberg (1944).

The ribitolphosphates obtained from both adenylic acids *a* and *b* (as well as from the guanylic isomers) by the steps shown in figure 13 are both optically inactive, indicating a 3′ structure (Doherty, '50). Unless one is willing to postulate phosphate migration, 2′, 3′ positional isomerism is ruled out by this finding. The results are more in keeping with α, β isomerism about the glycosidic linkage.

The question arises: what proof is there that these isomers exist within the parent nucleic acid molecule and that they are not formed by or during the alkaline hydrolysis? Ribonuclease hydrolysis yields only the *b* form of the cytidylic and

uridylic acids (Carter and Cohn, '50). Yeast ribonucleic acid, hydrolyzed by alkali, yields the 4 pairs with ratios of *a* to *b* of about 2:3 in each pair. (Figure 14 shows a complete analysis of such a hydrolysate.) The conditions of alkaline hydrolysis have been shown not to isomerize the individual nucleotides. These observations do not prove that the two isomeric forms coexist as such in the parent polynucleotide.

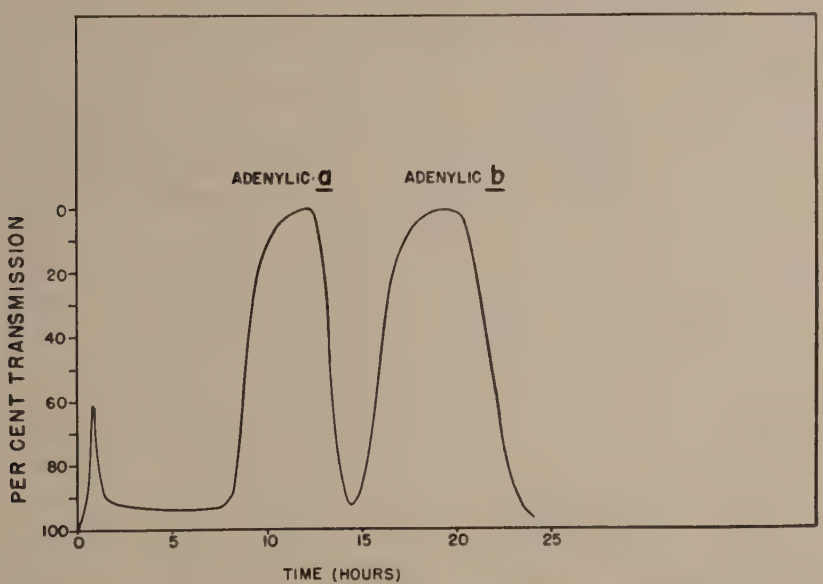

Fig. 12 Separation of ribose adenylic acids into two components.
Exchanger: Dowex-1, 250–500 mesh, 12 cm × 33 cm².
Eluting solution: 0.002 *M* HCl (pH 2.7) at 2.4–3.0 liters/hour.
Test material: 800 mg of ribose adenylic acids in 1000 ml at pH ~ 10.

DESOXYRIBONUCLEOTIDE SEPARATION. 5-METHYL-CYTIDYLIC ACID

Desoxyribonucleic acid (DNA) is resistant to chemical hydrolysis but yields to a combination of desoxyribonuclease and phosphatase to give a mixture of bases, nucleosides, nucleotides, and polynucleotide materials (Klein, '33; Klein and Thannhauser, '33, '34, '35). This mixture can be resolved by the same technique as is applied to the ribose compounds,

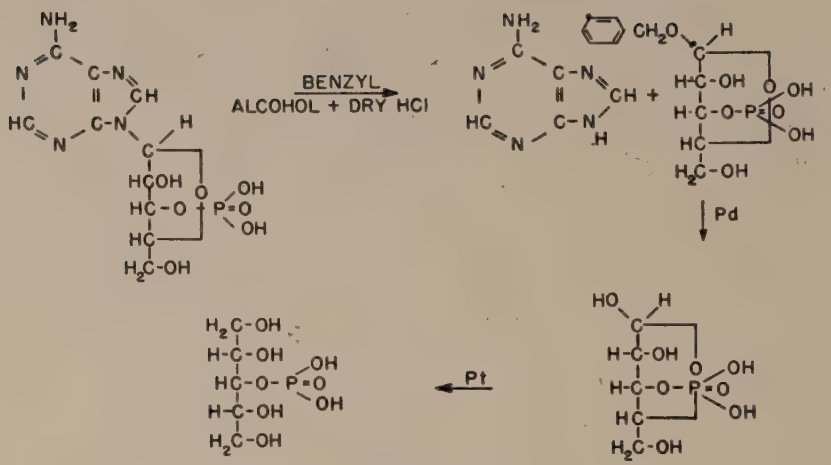

Figure 13

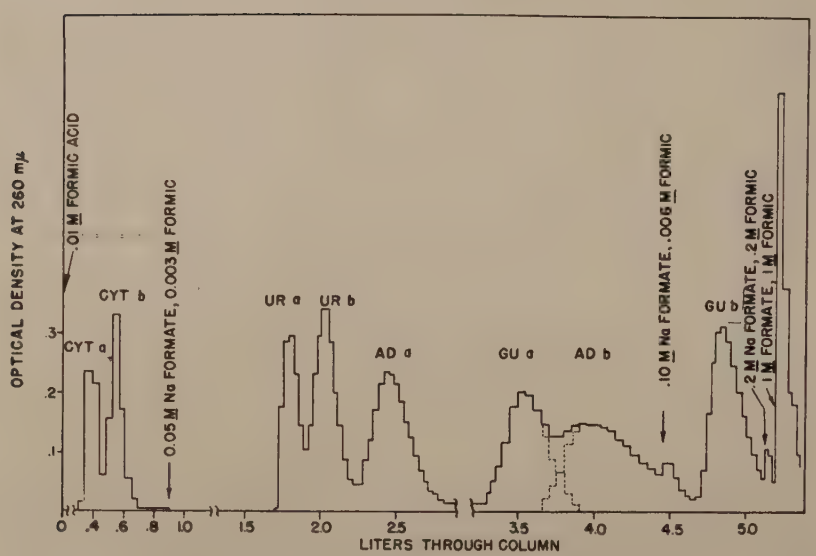

Fig. 14 Ion-exchange analysis of alkaline hydrolysate of yeast ribonucleic acid.

Exchanger: Dowex-1, 200–400 mesh, 12 cm × 0.82 cm², formate form.

Test material: About 20 mg purified yeast RNA 0.5 *N* NaOH, 37°C., 16 hours.

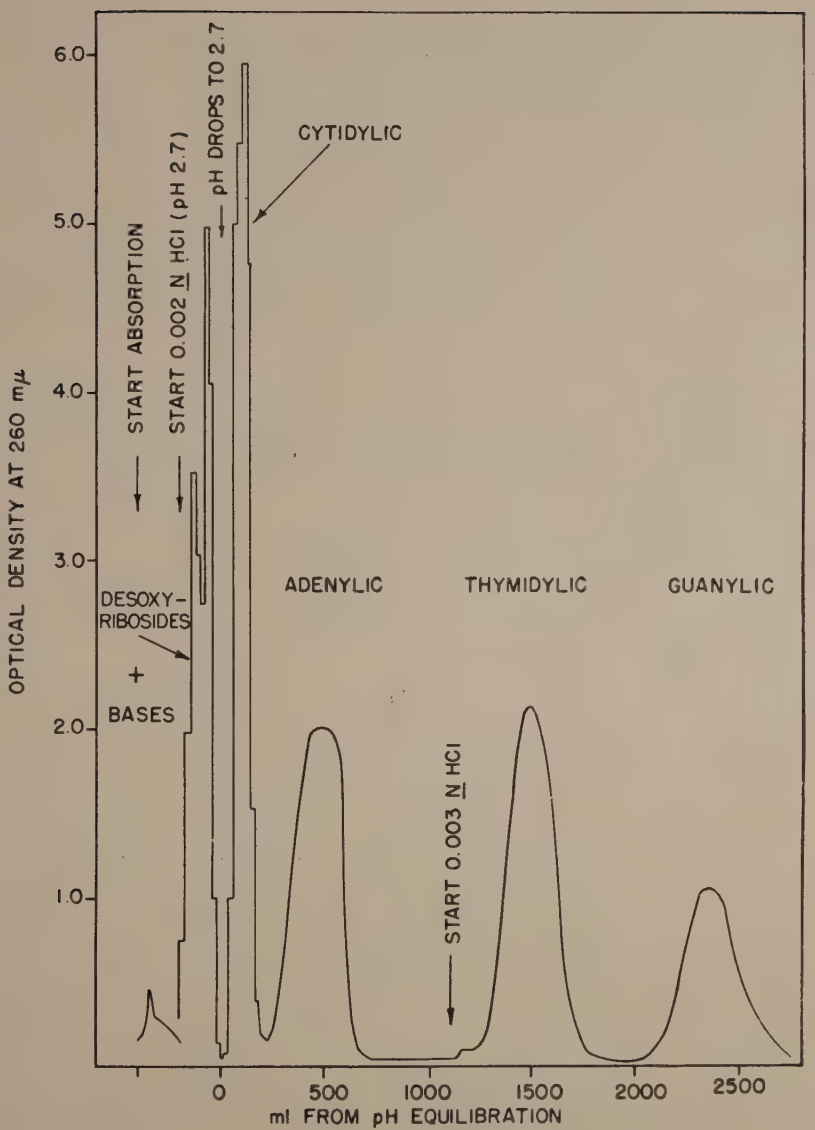

Fig. 15 Ion-exchange separation of desoxyribonucleic acid hydrolysate (150 mg).

Exchanger: Dowex-1, 200–400 mesh, 8 cm × 0.72 cm^2.

Eluting solution: HCl as shown, ca. 1 ml/min.

using slightly weaker acids to compensate for the lower distribution coefficients of the desoxyribonucleotides (see fig. 15, also first part of fig. 11). This separation has been carried out on the gram scale (Volkin, Khym, and Cohn, '51).

Careful analysis of the desoxycytidylic acid fraction indicates the presence of a second component present in small amount compared to the cytidylic acid (see X in fig. 11). This substance proved to be the desoxynucleotide of 5-methyl-

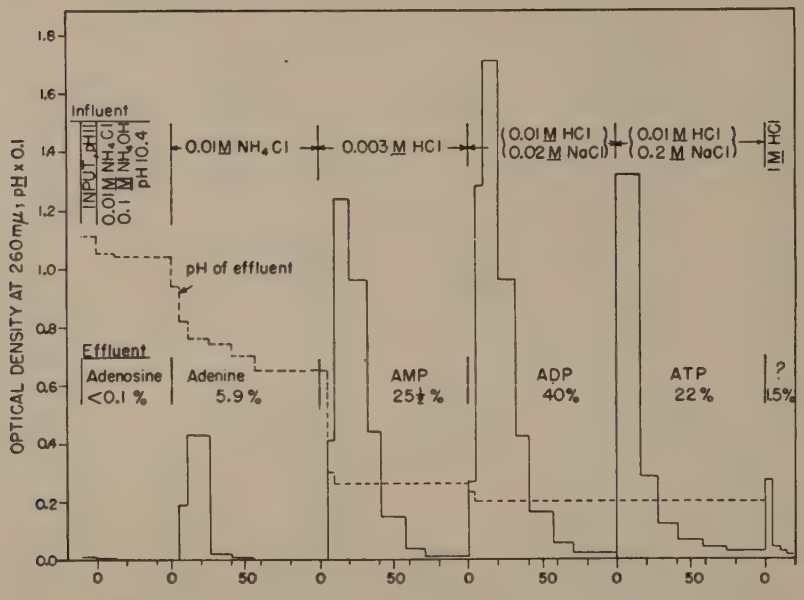

Fig. 16 Ion-exchange separation of adenosine phosphates.
Exchanger: Dowex-1, 200–400 mesh, 1 cm × 1 cm².
Test material: Commercial H_4ATP.

cytosine (Cohn, '50b, '51) recently reported to occur in many desoxynucleic acids (Wyatt, '50). Its isolation as a desoxypentose phosphate indicates that it should be considered a bonafide constituent of nucleic acids.

Comparison of figures 9 and 15 indicates that desoxyadenylic acid behaves identically with adenosine-5′-phosphate on the ion-exchange column. Desoxycytidylic acid and cyti-

dine-5′-phosphate, kindly supplied by Drs. Brown and Todd (Michelson and Todd, '49; Brown, Haynes and Todd, '50), likewise behave in an identical manner. This is consistent with enzymatic evidence indicating 5′-phosphate linkages in the desoxy series (Carter, '51).

ADENOSINE POLYPHOSPHATES

While not involved in nucleic acid structures, adenosine-diphosphate (ADP) and -triphosphate (ATP) are related to the nucleotides discussed above and their ion-exchange behavior becomes of interest. As might be expected, the presence of multiple acid groups markedly increases the distribution coefficient on anion exchangers. Hence, complete separations are easily achieved, as indicated in figure 16, which is the analysis of a commercial sample of ATP (Cohn and Carter, '50). This separation scheme has found immediate application in the preparation of ATP or ADP of high purity with respect to each other as well as other usual contaminants.

CONCLUSION

The application of ion exchange to nucleic acid chemistry has resulted in quantitative separation techniques for the nucleotides, nucleosides, and bases derived from nucleic acids. These techniques have, in turn, resulted in the discovery of isomers of all 4 ribose nucleotides and of desoxy-5-methylcytidylic acid. A method for the quantitative separation of adenosine di- and triphosphates has also been devised. It is proposed to apply the technique to the isolation of pure polynucleotides from enzymatic or chemical digests of nucleic acids.

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SOME RECENT STUDIES ON THE COMPOSITION AND STRUCTURE OF NUCLEIC ACIDS

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TEN FIGURES

The first question that must be answered before the determination of the structure of nucleic acids can be carried out in earnest is: how much of what do they contain? It seemed to us that procedures suitable for this purpose had been lacking in the past and that no real progress was possible, unless the necessary analytical groundwork was laid. Following the demonstration that the excellent method of chromatography on filter paper (Consden, Gordon and Martin, '44) could be applied to the separation of purines (Vischer and Chargaff, '47), procedures for the separation, in minute amounts, of the nitrogenous nucleic acid constituents and for their identification and quantitative estimation by spectrophotometry were elaborated (purines and pyrimidines: Vischer and Chargaff, '48a, b; ribonucleotides: Vischer, Magasanik, and Chargaff, '49; Chargaff, Magasanik, Doniger, and Vischer, '49; Magasanik, Vischer, Doniger, Elson and Chargaff, '50). Procedures for the hydrolysis of nucleic acids were discussed by Vischer and Chargaff ('48a, c), Chargaff, Vischer, Doniger, Green and Misani ('49), Chargaff, Magasanik, Doniger, and Vischer ('49), Chargaff, Magasanik, Vischer, Green, Doniger, and Elson ('50), and Zamenhof and Chargaff ('50b). Other applications of the methods, e.g., for the study of enzymatic reactions, also have been described (Chargaff and Kream, '48; Zamenhof and Chargaff, '49a; Kream and Chargaff, '50; Zamenhof and Chargaff, '50b). The results were recently reviewed by Chargaff ('50).

Before the conclusions reached by our group are briefly summarized, a few words should be said about the ultimate constituents of the nucleic acids studied by us. The preponderant nitrogenous constituents of the desoxypentose nucleic acids were the purines adenine and guanine, the pyrimidines cytosine and thymine; these components were present in varying proportions that were invariably quite far from the equimolar proportions required by a so-called tetranucleotide. Because of a previous claim of the presence of 5-methylcytosine in the nucleic acid of tubercle bacilli (Johnson and Coghill, '25), a special search for this component was made unsuccessfully by Vischer, Zamenhof and Chargaff ('49) in a desxoypentose nucleic acid preparation from avian tubercle bacilli (Chargaff and Saidel, '49). 5-Methylcytosine has, however, been recently shown by Wyatt ('50) to occur, mostly in minute amounts, in several desoxypentose nucleic acids from animal and plant cells. Furthermore, Cohn ('50) described a new desoxyribonucleotide component of calf thymus desoxyribonucleic acid (DNA) which, in collaboration with Kream and Zamenhof, could be shown to contain 5-methylcytosine as the only nitrogenous constituent (unpublished results).

With respect to the desoxy sugar component of nucleic acids, the comparison of the chromatographic behavior of the sugar components released from the desoxypentose nucleic acids from the following sources made it probable that they were, in all cases, identical with 2-desoxyribose: human thymus and liver; ox thymus, spleen, and liver; pig thymus; sheep thymus; salmon sperm; yeast; tubercle bacillus (partly unpublished results).

As regards the pentose nucleic acids, adenine, guanine, cytosine, and uracil, again in varying proportions, were the nitrogenous constituents found so far; and ribose was the only sugar identified in the nucleic acids from yeast, tubercle bacilli, human liver, pig liver and pancreas, ox and calf liver, and sheep liver.

DESOXYPENTOSE NUCLEIC ACIDS

The following conclusions, some necessarily provisional in nature, are drawn from the results of our work.

1. Desoxypentose nucleic acids are in their composition characteristic of the species from which they are derived. This can in many, but not in all, cases be demonstrated by determining the ratios in which the various purines and pyrimidines occur. There will, however, be many borderline cases in which these differences are not sufficiently significant to permit their use as the sole criterion of differentiation. (The problem of the differentiation between macromolecules of similar composition has recently been discussed in greater detail by Chargaff, '50.) The most important question of the sequence in which, in a particular nucleic acid, the nucleotides follow each other has barely been approached (Zamenhof and Chargaff, '50b). The importance of sequence analysis will be made clearer, if we consider that there can exist an enormous number of different nucleic acids giving the same analytical figures. For instance, a desoxypentose nucleic acid with the composition of that from calf thymus could exist in something like 10^{1500} permutations, if it consisted of 2500 nucleotides.

2. Different tissues of the same species yield the same desoxypentose nucleic acid. Here, the reservation must be made that this statement refers to the over-all composition, but not necessarily to the nucleotide sequence. No information is as yet available on the identity or difference of the latter in preparations from different organs of the same host.

3. The tetranucleotide hypothesis is incorrect. Not a single specimen analyzed so far approached in its proportions the analytical requirements even of a "statistical tetranucleotide." There appear, however, to exist several peculiar regularities. I think, we should formulate them not without trepidation, since we ought to avoid falling into a streamlined version of the old trap which in the past tripped so many excellent workers in the field of nucleic acid chemistry. It is quite possible that future work will show these generalizations to be unjustified. But as matters stand, it seems that

in most specimens examined until now the ratios of adenine to thymine, of guanine to cytosine, and of total purines to total pyrimidines were not far from one. Furthermore, two main groups of desoxypentose nucleic acids could be distinguished; in one group, adenine and thymine outweighed guanine and cytosine (the "AT type"); in the other group (the "GC type"), the converse was true. The last-mentioned type has so far been encountered only in certain microorganisms (Chargaff, Zamenhof, Brawerman, and Kerin, '50).

The composition of several preparations of desoxypentose nucleic acid from the organs of the ox is summarized in table 1 (Chargaff, Vischer, Doniger, Green, and Misani, '49; and

TABLE 1

Composition of desoxyribonucleic acid of ox (in moles of nitrogenous constituent per mole of P)

CONSTITUENT	THYMUS			SPLEEN		LIVER
	Prep. 1	Prep. 2	Prep. 3	Prep. 1	Prep. 2	
Adenine	0.26	0.28	0.30	0.25	0.26	0.26
Guanine	0.21	0.24	0.22	0.20	0.21	0.20
Cytosine	0.16	0.18	0.17	0.15	0.17	0.16
Thymine	0.25	0.24	0.25	0.24	0.24	0.25
Recovery	0.88	0.94	0.94	0.84	0.88	0.87

unpublished results). The composition of different samples of human desoxypentose nucleic acid (Zamenhof, Shettles, and Chargaff, '50) is similarly compared in table 2 (Chargaff, Zamenhof, and Green, '50; and unpublished results). Several desoxypentose nucleic acids of microbial origin are listed in table 3 (Vischer, Zamenhof, and Chargaff, '49; Chargaff, Zamenhof, Brawerman, and Kerin, '50; and unpublished results). The molar proportions are compared in table 4.

As has been pointed out, the search for methods permitting the determination of the sequence in which the nucleotides are aligned in a given nucleic acid appears to be very important. An orienting approach to this problem was made by Zamenhof and Chargaff ('50b) in a study of the course

TABLE 2

Composition of desoxypentose nucleic acid of man (in moles of nitrogenous constituent per mole of P)

CONSTITUENT	SPERM		THYMUS	LIVER	
	Prep. 1	Prep. 2		Normal	Carcinoma
Adenine	0.29	0.27	0.28	0.27	0.27
Guanine	0.18	0.17	0.19	0.19	0.18
Cytosine	0.18	0.18	0.16	0.15	0.15
Thymine	0.31	0.30	0.28	0.27	0.27
Recovery	0.96	0.92	0.91	0.88	0.87

TABLE 3

Composition of microbial desoxypentose nucleic acids (in moles of nitrogenous constituent per mole of P)

CONSTITUENT	YEAST		AVIAN TUBERCLE BACILLI	SERRATIA MARCESCENS	HYDROGEN ORGANISM, BACILLUS SCHATZ
	Prep. 1	Prep. 2			
Adenine	0.24	0.30	0.12	0.18	0.18
Guanine	0.14	0.18	0.28	0.24	0.26
Cytosine	0.13	0.15	0.26	0.28	0.29
Thymine	0.25	0.29	0.11	0.19	0.16
Recovery	0.76	0.92	0.77	0.89	0.89

TABLE 4

Molar proportions of purines and pyrimidines in desoxypentose nucleic acids from different species

SPECIES	ADENINE/GUANINE	THYMINE/CYTOSINE	PURINES/PYRIMIDINES
Ox	1.29	1.43	1.1
Man	1.56	1.75	1.0
Yeast	1.72	1.9	1.0
Avian tubercle bacillus	0.4	0.4	1.1
Serratia marcescens	0.7	0.7	0.9
Hydrogen organism	0.7	0.6	1.0

of degradation of calf thymus desoxypentose nucleic acid by crystalline desoxyribonuclease in which a decision was sought as to whether this nucleic acid was generally uniform throughout the molecule, i.e., whether it exhibited a recognizable regularity or periodicity of composition, or whether an even more complex pattern of the distribution of purine and pyrimidine nucleotides prevailed than could have been deduced as the simplest conclusion from the ultimate analytical findings. The latter was actually shown to be the case.

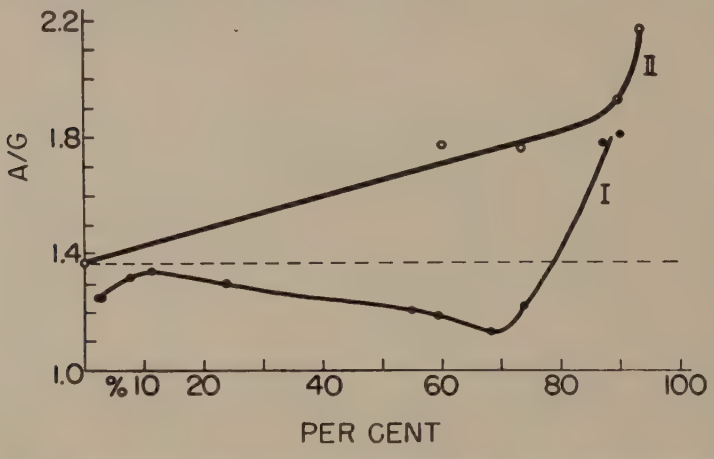

Fig. 1 Course of degradation of calf thymus DNA by crystalline desoxyribonuclease. Changes in the ratios of adenine to guanine in periodically collected portions of dialysate (curve I) and in total dialysis residue (curve II) in the course of enzymatic digestion and dialysis. The ratios are plotted as the ordinate; the abscissa idicates the degree of degradation of the DNA at the moment of sampling (expressed as per cent of original DNA found in total dialysate). The broken line represents the composition of the original DNA serving as substrate.

If one postulated an ideal case in which a desoxypentose nucleic acid exhibited ratios of adenine to guanine and of thymine to cytosine that were both 1.4 and ratios of adenine to thymine, of guanine to cytosine, and of purines to pyrimidines, all equalling one, a simple construction could, for instance, assume that a subunit consisting of 24 nucleotides contained 7 dinucleotides, in which adenylic acid was linked

to thymidylic acid, and 5 dinucleotides, in which guanylic and cytidylic acids were united, all these distributed in a certain pattern. The experimental results have, however, disproved this simplified assumption.

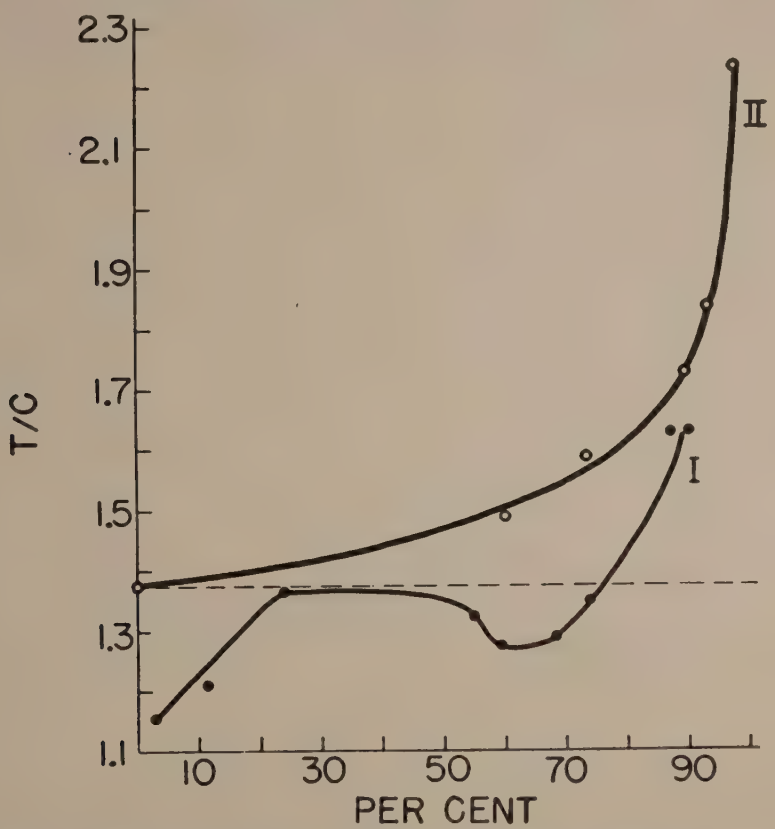

Fig. 2 Changes in the ratios of thymine to cytosine in dialysate (curve I) and dialysis residue (curve II). See figure 1 for other explanations.

When the action of crystalline desoxyribonuclease on calf thymus DNA was investigated and the composition of both the dialyzable fragments and the nondialyzable residue studied at different periods, it was found that a nondialyzable core persisted, whose composition differed very materially from that of the intact nucleic acid, and that the dialyzable

fragments varied characteristically with the extent of digestion, thus suggesting a very complex pattern of the sequence of individual nucleotides in the original nucleic acid. The core was characterized by greatly increased ratios of adenine to guanine, thymine to cytosine, purines to pyrimidines, and by greater resistance to enzymatic attack (Zamenhof and

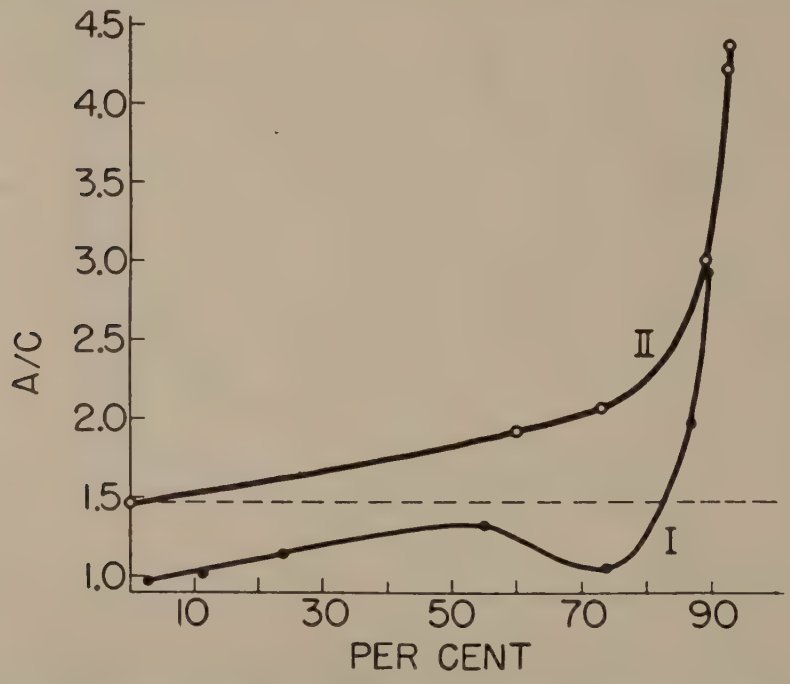

Fig. 3 Changes in the ratios of adenine to cytosine in dialysate (curve I) and dialysis residue (curve II). See figure 1 for other explanations.

Chargaff, '49a, '50b). Some of these results are illustrated in figures 1 to 4. Table 5 reproduces some of the figures obtained in a representative experiment.

Other recent studies dealt with the depolymerization behavior of desoxypentose nucleic acids (Zamenhof and Chargaff, '50a). When the stabilities toward acid of the DNA of yeast (Chargaff and Zamenhof, '47, '48) and of calf thymus DNA were compared, significant differences were discovered.

While the limit of stability to acid of calf thymus nucleic acid was found at pH 5.8, as shown by a gradual viscosity drop at lower pH values, the corresponding preparation from yeast was entirely stable down to pH 3.7 and depolymerized extremely rapidly below this value. (Compare figs. 5 and 6.) No such clear-cut distinctions can, however, be found on the alkaline side (fig. 7).

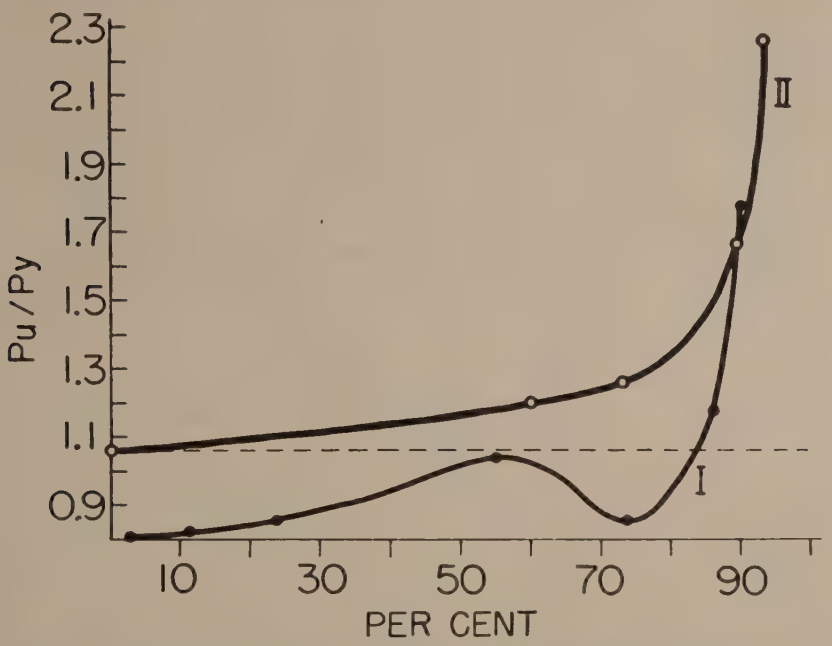

Fig. 4 Changes in the ratios of purines to pyrimidines in dialysate (curve I) and dialysis residue (curve II). See figure 1 for other explanations.

TABLE 5

Enzymatic degradation of calf thymus desoxyribonucleic acid

	DIGESTION	DIALYSIS	DISTRIBUTION OF FRACTIONS % OF ORIGINAL	COMPOSITION OF FRACTIONS (MOLAR PROPORTIONS)			
				ADE./GUA.	THY./CYT.	ADE./CYT.	PUR./PYR.
	hours	*hours*					
Original	0	0	100	1.2	1.3	1.6	1.2
Dialysate	6	6	53	1.2	1.2	1.2	1.0
Dialysis residue	24	72	7	1.6	2.2	3.8	2.0

Another problem, studied on the same occasion (Zamenhof and Chargaff, '50a), had to do with the problem of the native state of desoxypentose nucleic acids and with what may be considered as their denaturation. Although neutralized solu-

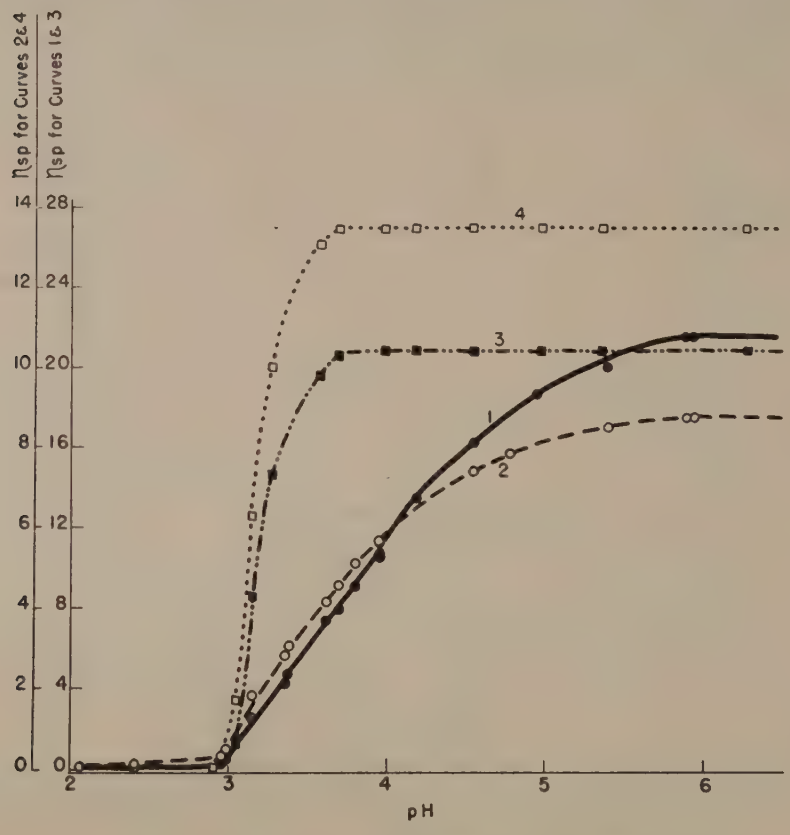

Fig. 5 Stability of calf thymus and of yeast desoxypentose nucleic acids to acid. The solutions in 0.05 *M* aqueous NaCl had the following concentrations: calf thymus DNA, 0.25% (curves 1 and 2); yeast DNA, 0.39% (curves 3 and 4). The specific viscosities are plotted as the ordinate; the abscissa indicates the pH. Curves 1 and 3, Ostwald-Fenske viscosimeter under gravity; curves 2 and 4, under constant applied pressure.

tions of nucleic acid (after degradation by acid, alkali, or heat) are able to regain high viscosity, it could be shown that the aggregates formed were artifacts which had a highly

thixotropic character (fig. 8), in contrast to the constant viscosity of the intact preparations, and which also differed in thermal stability (fig. 9).

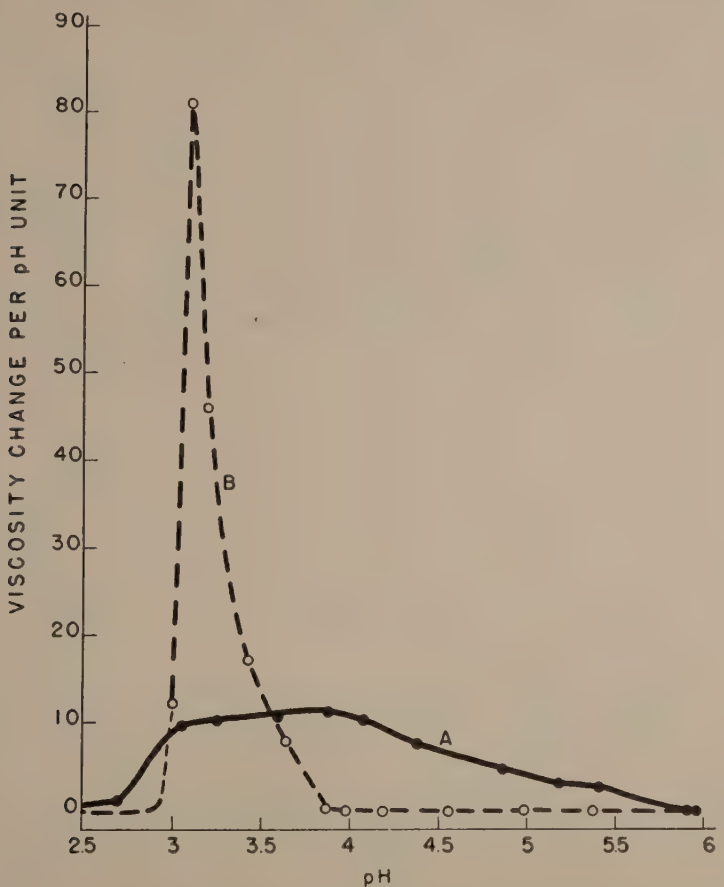

Fig. 6 The viscosity changes under gravity per pH unit (viscosity gradients) are plotted as the ordinate, the pH values as the abscissa. Curve A, calf thymus DNA; curve B, yeast DNA.

No more than brief reference can be made here to studies of the desoxypentose nuclease of yeast cells and to the specific nature of its inhibition (Zamenhof and Chargaff, '48, '49b).

PENTOSE NUCLEIC ACIDS

Though at least one pentose nucleic acid, namely that of yeast, has been studied more extensively and by a greater number of workers than were the desoxypentose nucleic acids,

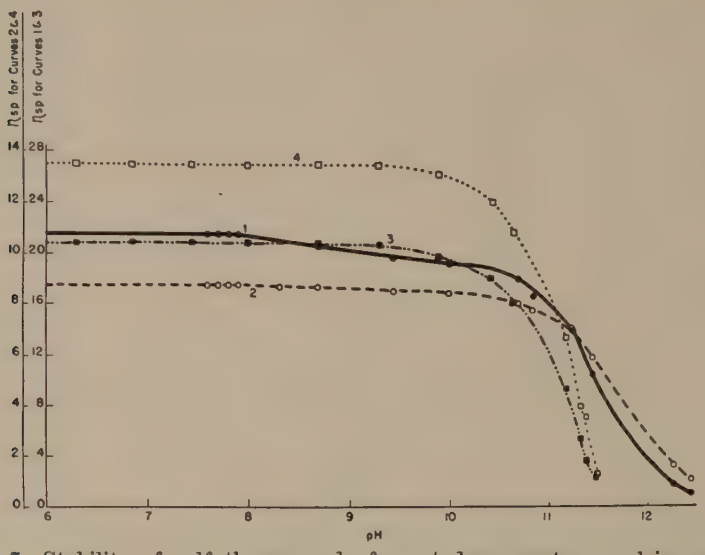

Fig. 7 Stability of calf thymus and of yeast desoxypentose nucleic acids to alkali. See figure 5 for other explanations.

one can only feel that less is known about the pentose-containing acids. Both the abundance of papers on this subject and the unsatisfactory state of our knowledge can, at least in part, be attributed to the fact that yeast ribonucleic acid (RNA) has long been available commercially, often in a lamentable state of preservation. The ease with which material, carrying the designation of nucleic acid, could be procured served to throw a cloak of uniformity over the entire nucleic acid field. Actually, no entirely satisfactory methods for the preparation of pentose nucleic acids are as yet available.

A consideration of the findings on the composition of pentose nucleic acids from different cellular sources should be preceded by an attempt to answer a number of questions that

will arise whenever the structure of a highly polymerized substance of natural origin is to be considered, such as: are the samples studied representative? Are the results obtained

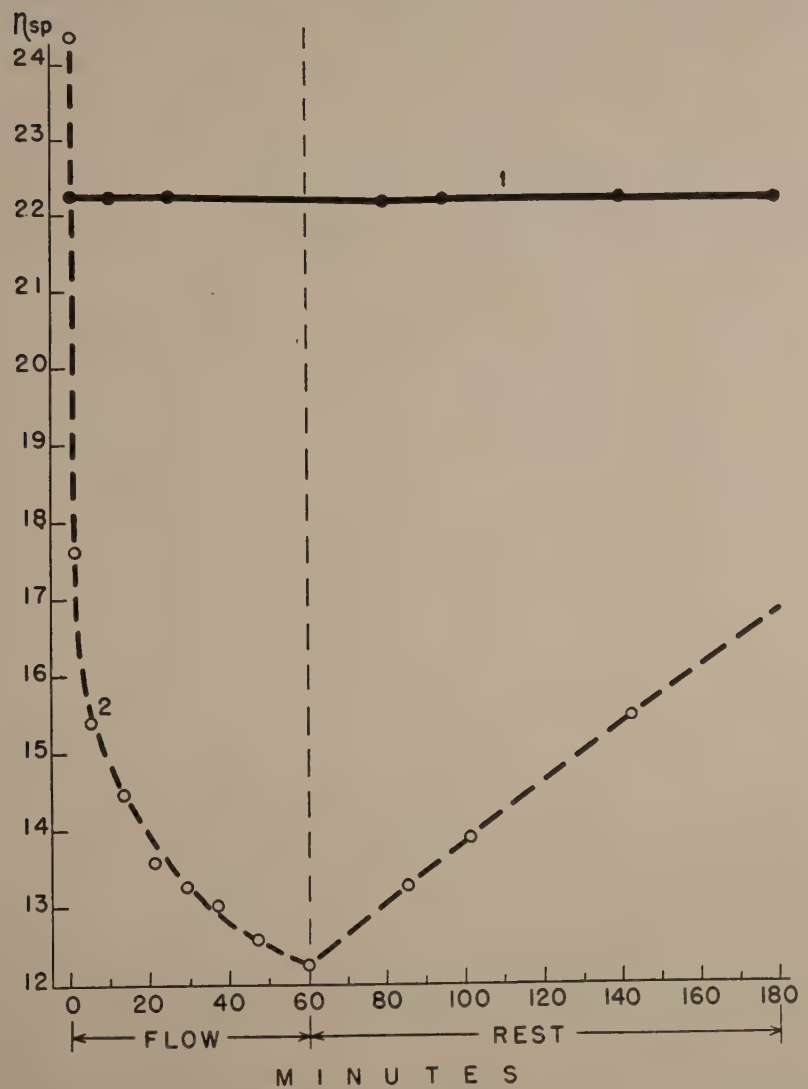

Fig. 8 Nonthixotropic viscosity of undegraded (curve 1), thixotropic viscosity of degraded (curve 2) calf thymus desoxypentose nucleic acid.

with them uniform? Are the methods used in the study reliable?

The question of representativeness is closely connected with our present lack of knowledge of the criteria decisive for the identity or diversity of high-molecular cell constituents composed of many molecules of a limited number of

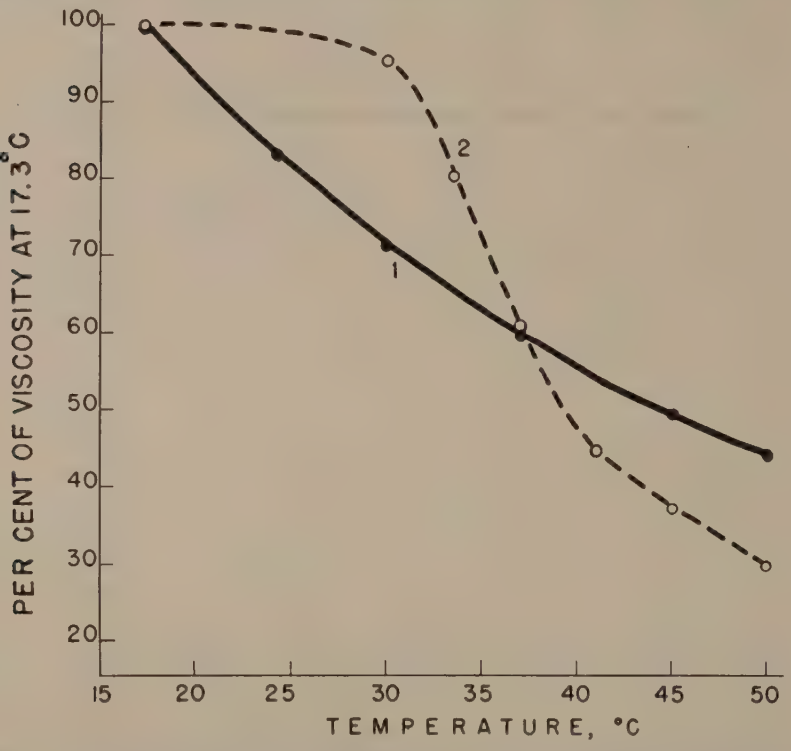

Fig. 9 Viscosity behavior of undegraded (curve 1) and heat-degraded (curve 2) calf thymus desoxypentose nucleic acid as a function of the temperatures of measurement.

organic substances and can at present be answered in the affirmative in almost no case, with the possible exception of very few enzymes and hormones of clearly circumscribed origin and function. This problem has been treated at greater length in a recent article (Chargaff, '50). Here, it will suffice to point to the difficulty of a decision, even on the most

primitive level, as regards the two types of nucleic acid occurring in cells. Extranuclear desoxypentose nucleic acid does not seem normally to be present in the majority of mammalian cells; this substance or mixture of closely related substances is limited to the chromatin material of the cell nucleus or, in certain stages of cellular development, to a number of well-defined structures, the chromosomes. Even in this case it is often difficult to affirm that we are dealing with one molecular species only, though it is derived from the same cell. This difficulty becomes much greater in the case of the animal pentose nucleic acids. Pentose polynucleotides of undetermined molecular size are known to occur in many parts of the cell, e.g., the mitochondria, the submicroscopic particles, the nucleolus. Whether they are identical or whether they differ in the proportion and sequence of their components, cannot yet be said; but it is hoped that studies undertaken by us at present will shed some light on the question as to whether it is possible to speak of the pentose nucleic acid of a particular organ or of a particular species. In this respect, it will be very long before work of this kind will lose its almost inherently provisional quality. What has been said here of the animal pentose nucleic acids applies, more or less, also to the RNA of yeast.

The question as to the uniformity of results refers, of course, to the same general problem, namely whether different specimens of pentose nucleic acid isolated from the same cellular source have an identical composition. The absence of this feature could be due to a lack in the uniformity of pentose nucleic acid, as it occurs in different parts of the cell or at different periods of cellular development, in which case the isolation of the preparations would presumably be accompanied by a certain amount of fractionation. This question can be answered tentatively in a few cases. Four yeast preparations studied by us, though isolated by different procedures, had a rather similar composition. On the whole, the differences observed lend no support to the assumption that changes in the preparatory methods led to

the isolation of mixtures of widely divergent composition. Similarly, preparations from calf and beef liver were in all important features more similar to each other than they were to specimens from the liver of other species.

In this connection a few words should be said about the quality of pentose nucleic acid preparations available for study which, unfortunately, is far from perfect in most cases. Whereas it is now possible to extract the bulk, if not all, of the desoxypentose nucleic acid of a cell by mild and relatively innocuous procedures, this is not true of the pentose nucleic acids, if representative, i.e., truly comprehensive, preparations are desired. Different portions of the cell appear to hold pentose nucleic acid with different degrees of tenacity; the milder the method of isolation is, the greater is, therefore, in this case the danger of concomitant fractionation. Although the pentose nucleic acid of a cell, if it is possible to speak of such an entity, may eventually prove an even more fragile substance than is desoxypentose nucleic acid, almost all isolation procedures call for a comparatively drastic treatment.

The analytical studies of pentose nucleic acid composition reported here made use of three methods: (1) estimation of all the ribonucleotides following the cleavage of the specimen with alkali under mild conditions; (2) estimation of the purines as the free bases and the pyrimidines as the nucleotides; (3) estimation of all nitrogenous constituents as the free bases (Vischer and Chargaff, '48b, c; Chargaff, Magasanik, Doniger, and Vischer, '49; Chargaff, Magasanik, Vischer, Green, Doniger, and Elson, '50). When these three methods are evaluated critically, it is found that methods 1 and 3 yield the most reliable values for purines, whereas for pyrimidines methods 1 and 2 are preferable.

The most convenient procedure for the study of the composition of pentose nucleic acids appears one similar to method 1 mentioned before, which is based on the cleavage of the nucleic acid to mononucleotides at pH 13 to 14 and 30°C. The separation of mononucleotides by paper chromatography

in a solvent system consisting of aqueous isobutyric acid — ammonium isobutyrate and the spectrophotometric estimation of the separated components have been described in detail by Magasanik, Vischer, Doniger, Elson, and Chargaff ('50). As a supplement to the discussion, given by these authors, of the conditions underlying the chromatographic separation

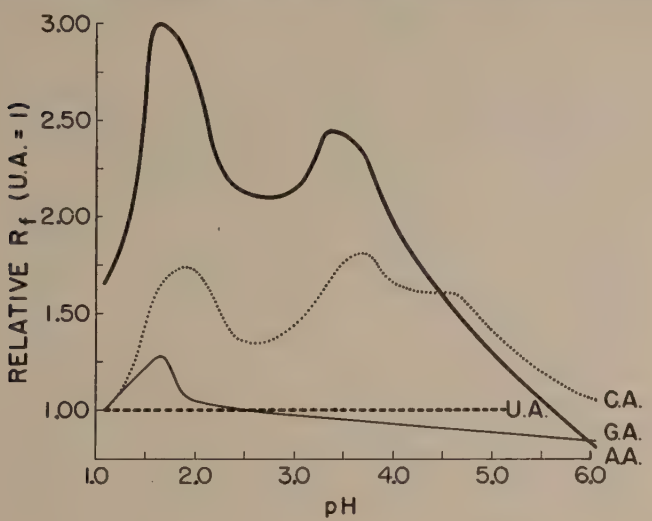

Fig. 10 Relative R_f values of the ribonucleotides as a function of the pH of the solvent system, with the R_f of uridylic acid taken as 1. G.A. — guanylic acid; A.A. — adenylic acid; C.A. — cytidylic acid; U.A. — uridylic acid.

of the nucleotides which, with the exception of uridylic acid, are polyvalent ampholytes, figure 10 is reproduced here, showing the relative R_f values of the three nucleotides capable of occurrence as dipolar ions, with the position of uridylic acid, the only nonampholyte, taken as the reference point at the respective pH. The choice of this nucleotide as the reference substance is due to the fact that since its charge characteristics change very little between pH 2 and 5, a sharper definition of the contribution made by changes in ionization of the other nucleotides becomes possible. Below pH 2, uridylic acid, probably, is not suitable as a reference compound.

The results on the nucleotide composition of a number of pentose nucleic acid preparations from yeast and animal tissues, reproduced in table 6, are taken from a recent publication (Chargaff, Magasanik, Vischer, Green, Doniger, and Elson, '50). They show that, in contrast to yeast RNA, the preparations from animal tissues were characterized by a high proportion of guanylic acid and, in most of the fractions from liver, also of cytidylic acid.

TABLE 6

Nucleotide composition of pentose nucleic acids; molar relationships

PREPARATION NO.	SOURCE	GUANYLIC ACID	ADENYLIC ACID	CYTIDYLIC ACID	URIDYLIC ACID	PUR./PYR.
1	Yeast	9.7	10	6.1	7.0	1.5
2	Yeast	9.6	10	7.5	6.7	1.4
3	Yeast	10.6	10	8.6	8.2	1.2
4	Yeast	10.5	10	8.0	10.2	1.1
5	Pig pancreas	22.5	10	9.8	4.6	2.3
6	Pig liver	16.3	10	16.1	7.7	1.1
7	Sheep liver	16.8	10	13.4	5.6	1.4
8	Calf liver	16.2	10	11.1	5.3	1.6
9	Beef liver	14.6	10	10.9	6.6	1.4
	Carcinomatous human liver					
10	Unaffected tissue	32.9	10	28.8	8.3	1.1
11	Metastases	41.4	10	43.2	7.2	1.0

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ON THE NATURE OF THE PRODUCTS FORMED BY THE ACTION OF CRYSTALLINE RIBONUCLEASE (KUNITZ'S RIBONUCLEASE) ON YEAST RIBONUCLEIC ACID[1]

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In the history of nucleic acid chemistry, methods of partial hydrolysis have been of particular significance, due to the structure of the component nucleotide groups. Not only the interlinkages between these component units, but also those between the three different groups of each component unit are hydrolyzable bonds. A cleavage of nucleic acid molecules to simpler polynucleotides is, therefore, only possible if the hydrolysis can be conducted in such a manner that the mononucleotide groups remain intact — a problem which has relatively few analogies in the chemistry of other cell constituents of high molecular weights such as proteins and polysaccharides.

The application of alkali and acid hydrolysis by the pioneer investigators, by Kossel, by Levene, and by Thannhauser and their collaborators ('31) has led to the almost complete elucidation of the structure of the component mononucleotides. But even during this phase of nucleic acid chemistry, during the study of the mononucleotides, it became clear that the hydrolyzing agents of the chemical laboratory were too drastic and too unspecific for many problems requiring methods of partial hydrolysis. Thus, the isolation of the nucleosides and nucleotides of thymonucleic acid and the elucidation

[1] These studies were aided by grants from the Rockefeller Foundation, the American Cancer Society, Inc., the Godfrey H. Hyam's Trust Fund, the Bingham Association, and the Charlton Fund.

of the nature of the carbohydrate groups of thymonucleic acid was only possible after Levene, Mikeska, and Mori ('29, '30) and Bielschowsky and Klemperer ('32, '33, '34) in Thannhauser's laboratory had turned to the application of enzymes as hydrolyzing agents.

The insufficiency of the hydrolyzing laboratory reagents becomes a decisive difficulty for the experimental approach to the question of the manner in which the mononucleotides are linked with each other in the nucleic acid molecule. Except for thymic acid which is by no means a well-defined substance, no degradation product whose molecular size is between those of the nucleic acids and those of the mononucleotides has so far been obtained by chemical hydrolysis. It is illustrative to contrast this situation in the nucleic acid field with the considerable number of peptides which could be isolated from chemical hydrolysates of proteins.

The attempt to apply enzymes as tools for the partial degradation of the genuine nucleic acids is not new. More than 30 years ago, Thannhauser ('17) found that the incubation of yeast ribonucleate with duodenal juice did not lead to the complete cleavage of the interlinkages between the mononucleotides, but resulted in the formation of polynucleotides. Walter Jones ('20, '23) discovered the presence in pig's pancreas of a heat-stable enzyme system which catalyzed the hydrolysis of ribonucleic acid (RNA) into mononucleotides which were identical with the mononucleotides obtained by chemical hydrolysis of RNA. The investigations of Takahashi ('32) concerning the existence of enzymes specific for the hydrolysis of phosphomono- and diester groups, respectively, further stimulated the application of enzymes to investigations concerning the nature of the phosphoric acid bonds in nucleic acids. On the basis of the resistance of yeast RNA against phosphomonoesterases, Takahashi ('32) suggested the assumption that yeast ribonucleic acid contained no phosphomonoester groups and consisted of a cyclic chain of mononucleotides. Despite the fact that the observations of Takahashi concerning the specificity of his phospho-

mono- and diesterases could not be reproduced by Klein ('35) the differentiation between mono- and diesterase activity of phosphatases proved to be a useful working hypothesis in subsequent investigations by other authors (Bolomey and Allen, '42; Schmidt et al., '47; Gulland and Jackson, '39).

The most important progress in the study of nucleic acid splitting enzymes was achieved when Kunitz ('40) succeeded in crystallizing ribonuclease, a component of the nucleolytic enzyme system of the pancreas, and of desoxyribonuclease ('50) whose presence in pancreas had been discovered 12 years earlier by Feulgen ('36). Allen and Eiler ('41) and Bain and Rusch ('44) demonstrated that the action of ribonuclease consisted specifically in the hydrolysis of phosphodiester groups of ribonucleic acids to phosphomonoester groups. Bolomey and Allen ('42) found that preliminary treatment of ribonucleic acid with ribonuclease strongly enhanced the enzymatic dephosphorylation of the nucleotide groups by phosphatase of almond extracts.

The interpretation of these observations was complicated by the fact that the almond extract used contained other enzymes besides phosphatase. We found that it was of advantage to replace the almond extract of Bolomey and Allen's enzyme system by purified preparations of prostate phosphatase. Even these could not as yet be obtained without small contamination with ribonuclease; the exceptionally high phosphatase activity of prostate preparations made it possible, however, to find conditions of incubation under which the phosphatase action of the enzyme greatly predominated over the other enzymatic activities of the solutions.

The application of enzymes as tools for the analysis of nucleic acid hydrolysates is more complicated than the use of these agents in the well-known classical problems of structural chemistry such as the biological resolution of racemic mixtures or the stereochemistry of glucosides or the enzymatic determinations of metabolites such as urea, uric acid, guanine, and 5-adenylic acid. In these cases, the specificity of the enzymes and the completion of the enzyme reaction

can easily be demonstrated, and the substrates were substances of comparatively simple structures. The problem of the present investigation is much more complicated and has been adequately described in an unpublished comment of the late J. M. Gulland as an attempt to obtain information "on a substance of unknown structure by the application of an enzyme of unknown specificity." It is, therefore, not superfluous to mention briefly some general rules which must be observed for the application of enzymes as tools for the approach of structural problems.

End point of enzyme reactions. The quantitative partition of chemical groups by specific enzyme reactions is only possible if it can be assumed that the end point of the enzyme reaction is caused exclusively by the exhaustion of the groups susceptible to the enzymatic cleavage. This implies that the possibility of a pseudo end point caused by inhibiting influences on the enzymes must be excluded by kinetic studies. Another practical consequence is the necessity of the use of powerful enzyme preparations under optimal conditions of activity. Some similar studies of the past have led to inconclusive results due to the use of weakly active enzyme solutions.

The use of strong enzyme preparations entails the danger that even small contaminations of the specific enzyme with other enzymes might cause undesirable side reactions to an appreciable extent. Only the analysis of the enzymatic digest with independent methods can show if the occurrence of enzymatic side reactions has to be considered in the interpretation of the results.

Specificity of prostatic phosphatase. The available preparations of prostatic phosphatase had favorable though not ideal properties for the partition of organic phosphorus containing groups of nucleic acid hydrolysates. They hydrolyzed all mononucleotides isolated from hydrolysates of ribonucleic acids as well as monophenyl phosphate at very similar rapid rates. (Other phosphoric monoesters such as adenosine-5-phosphate and ribose-5-phosphate were hydrolyzed at approxi-

mately one-third the rate of adenosine-3-phosphate, Robison-ester at one-fifth this rate.) On the other hand, phosphoric diesters such as diphenyl phosphate, glycerylphosphorylcholine, and glycerylphosphorylethanolamine were completely resistant against the action of prostate phosphatase.

Nevertheless, these enzyme preparations, at least in their present state of purity, were not suitable for the differentiation of phosphoric mono- and diester groups in genuine ribonucleic acids because they contained small amounts of the ribonuclease which could be easily demonstrated when the monoesterase activity was destroyed by heating to 100°C. during 15 minutes. When ribonucleate was incubated with heated prostate extracts, no inorganic phosphate was formed, but the amount of organic phosphate compounds soluble in MacFadyen's reagent ('34) (1.5% uranyl chloride in 10% trichloroacetic acid) increased slowly. Since RNA is entirely insoluble in MacFayden's reagent, this behavior indicates the presence of a heat-stable nuclease in prostate extracts.

When RNA was incubated with prostate phosphatase during relatively short periods (2–8 hours) only a small percentage of its total phosphorus groups (5–10%) was transformed into inorganic phosphate. The dephosphorylation which started with considerable speed decreased soon to a very slow rate but did not come to a complete standstill. Thus, the ribonuclease activity of the phosphatase preparations rendered impossible their use for the determination of the phosphoric monoester groups of genuine ribonucleate.

Hydrolysis of RNA by ribonuclease and prostate phosphatase. It appeared feasible, however, to achieve a specific enzymatic dephosphorylation of the phosphomonoester groups of RNA after its exhaustive depolymerization with ribonuclease. It was found that a digest obtained by incubation of yeast RNA with excessive amounts of ribonuclease was hydrolyzed by prostate phosphatase at much more rapid rates than genuine yeast RNA. The formation of inorganic phosphate came to a standstill after approximately 45% of the total phosphate of the digest had been transformed into inorganic

phosphate. The maximal degree of hydrolysis was independent of the amounts of phosphatase used.

The inorganic phosphate originated almost exclusively from the pyrimidine nucleotide groups of the digest. The dephosphorylation of the pyrimidine groups was 93% under optimal conditions of incubation.

These conclusions were based on two independent observations. (1) Ninety-seven per cent of the organic phosphorus of the digest was hydrolyzable by 1.5 *N* sulfuric acid at 100°C. within 60 minutes. The amount of the hydrolyzable phosphorus compounds of the enzymatic digest was practically the same as that present in the digestion mixture before the incubation. (2) Only negligible amounts of purine compounds were found after the removal of all phosphoric acid compounds from digests obtained from RNA by subsequent action of ribonuclease, phosphatase, and alkali.

The simplest explanation for the specific dephosphorylation of the pyrimidine groups is the assumption that ribonuclease specifically hydrolyzes nearly all interlinkages between the pyrimidine nucleotide groups of RNA under preservation of the interlinkages of the purine nucleotide groups. This is only possible if no alternating sequence of simple purine and pyrimidine nucleotide groups occurs in the RNA molecule, and if RNA consists of chains of purine mononucleotides linked to chains of pyrimidine mononucleotides.

Consequently, one would expect that the hydrolysis products of the action of ribonuclease are two types of nucleotides: (1) pyrimidine mononucleotides and (2) purine polynucleotides containing single pyrimidine mononucleotides predominantly as terminal groups. Polynucleotide fractions of the type mentioned under (2) have actually been isolated from ribonuclease digests by Schmidt, Cubiles, Swartz, and Thannhauser ('47). Information concerning the mutual proportion of these two types of hydrolysis products could be obtained by determining the amounts of nucleosides in the digest. For this purpose, the nucleosides were quantitatively separated

from the phosphorus containing substances of the digest by precipitation with uranyl acetate in presence of inorganic phosphate. The amounts of nucleosides in the supernatant were quantitatively determined by oxidation with periodate. It was found in control experiments that each of the 4 ribonucleosides consumed one equivalent of periodate. In comparison with techniques based on N determinations or absorption spectra, the application of the periodate for the analysis of the pyrimidine nucleosides has the advantage that equivalent amounts of cytidine and uridine give identical titration figures. It was found with this technique that, after exhaustive hydrolysis of yeast RNA with ribonuclease, close to 60% of the pyrimidine nucleotide groups had been split off as mononucleotides, whereas the other half of the pyrimidine groups were present as terminal groups of purine polynucleotides. Purine-containing mononucleotides were practically absent in the digest. The absence of appreciable amounts of purine mononucleotides in ribonuclease digests of yeast RNA has recently been established by Carter and Cohn ('50) by chromatographic analysis.

Yeast RNA and its digests with ribonuclease do not consume any periodate prior to their incubation with phosphatase. This shows that all nucleotide groups in genuine RNA, as well as in the ribonuclease digests, are esterified with phosphoric acid either in position 2′ or in position 3′ — in agreement with the structure suggested by Levene on the basis of the results obtained by chemical hydrolysis methods.

It was found, however, that after incubation with phosphatase, periodate was consumed not only by the nucleoside fraction, but also by the polynucleotides precipitated with uranyl acetate. The amount of total periodate consumed was approximately equivalent to the amount of inorganic phosphate formed. This suggests that at least the terminal pyrimidine nucleotide groups formed by the action of the ribonuclease has been linked with the neighboring nucleotide

groups at positions other than 2′- or 3′-positions of the ribose groups of the original RNA.

There is evidence that the mechanism of ribonuclease action as suggested by the observations just discussed is not the only pathway of the enzymatic hydrolysis of yeast RNA. Whereas crystalline ribonuclease hydrolyzes exclusively interlinkages between the pyrimidine nucleotide groups, crude pancreas and spleen extracts, as well as the almond extracts studied by Bolomey and Allen ('42), act on both the pyrimidine as well as the purine nucleotide interlinkages. Additional support for the existence of different ribonucleases with different specificity were obtained when polynucleotide fractions obtained by exhaustive digestion of RNA with crystalline ribonuclease were used as substrate instead of RNA itself. These polynucleotides were resistant against further incubation with crystalline ribonuclease. They were hydrolyzed, however, during the incubation with enzyme preparations from beef spleen which had been freed of phosphatase. Maver and Greco ('49) found recently that the nucleolytic action of spleen extracts differed in regard to their pH optimum from that of Kunitz's crystallized ribonuclease. Similarly, we found that spleen extracts which hydrolyzed vigorously the purine polynucleotide fractions obtained by the action of crystalline ribonuclease were inactive in bicarbonate systems of a pH of 7.5. The maximal extent of hydrolysis so far obtained amounted to 50% of the nucleotide groups of the polynucleotide.

The existence of polynucleotidases whose specificity differs from that of Kunitz's ribonuclease would explain several observations which cannot be understood on the basis of the specificity of crystallized ribonuclease. Jones ('20) found that the enzymatic hydrolysis of RNA by pancreas extracts leads to the formation of both purine- and pyrimidinenucleotides. Bredereck ('38) and Bolomey and Allen ('42) obtained all 4 ribonucleosides by incubating ribonucleic acid with extracts from sweet almonds. Loring and Carpenter ('43)

isolated all 4 mononucleotides from digests of ribonucleic acid with crystalline ribonuclease.

SUMMARY

1. Kunitz's ribonuclease specifically hydrolyzes at least 93% of the interlinkages between the pyrimidine nucleotide groups of yeast ribonucleic acid.

2. An alternating sequence between individual purine and pyrimidine nucleotides, or between pairs of purine nucleotides and pairs of pyrimidine nucleotides, such as required by the tetranucleotide concept of the structure of yeast ribonucleic acid, has been excluded.

3. Yeast ribonucleic acid consists of chains of purine nucleotides, which are linked to chains of pyrimidine nucleotides of an average length of two to three individual pyrimidine nuleotides.

These conclusions concern the largest part of the pyrimidine nucleotide groups and do not exclude a different mode of linkage for a relatively very small number of pyrimidine nucleotide groups.

4. A considerable number of interlinkages between the pyrimidine nucleotides of ribonucleic acid involves groups other than the 2 to 3 groups of the ribosyl groups.

5. Some observations suggesting the existence of several ribonucleases with different specificities have been reported.

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THE DISTRIBUTION AND INTERRELATION OF NUCLEIC ACIDS IN FIXED CELLS AS SHOWN BY ENZYMATIC HYDROLYSIS[1]

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TWENTY-FOUR FIGURES

The cytological approach to problems of cell chemistry involves as the first step a redefinition of morphological concepts in chemical terms. Descriptions in terms of discernible structure are accordingly being supplemented by descriptions in terms of constituent nucleic acids, proteins and associated materials. Since the nondividing cell presents a relatively constant pattern of visible structural detail, it has served as the focus of a number of quantitative as well as qualitative studies of cellular organization. The dividing cell, on the contrary, exhibits a changing series of morphological states incident to the movement and separation of the chromosomes, and these cyclic transformations complicate methods of chemical analysis. Nevertheless the dividing cell presents the greater challenge as well as the greater complexity, for the maneuvers of mitosis reflect basic processes of chromosome duplication and cell differentiation. Ultimately these processes must be analyzed in terms of the fundamental chemical structure of the living material as it changes in the course of mitosis and in the reparative and synthetic processes characteristic of its metabolic states.

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Although attainment of these objectives lies at present beyond the scope of cytochemistry, some progress toward the desired goal should be possible by applying techniques that permit recognition and location of cellular components, that indicate their patterns of association, and provide estimates of their quantitative relations. With these prospects in mind we initiated a few years ago a series of experiments using purified enzymes in association with staining procedures to determine the distribution and interrelations of nucleic acids and proteins in a variety of plant and animal cells. Actively growing tissues were chosen in whose cells clearly defined chromosomes could be readily recognized; in other words the classical materials of the descriptive cytologists were employed, such as root tips and anthers of onion, lily, *Tradescantia,* and *Vicia,* spermatogenous cells of grasshoppers, and, for certain types of corroborative evidence, the giant chromosomes of the salivary glands of *Drosophila* and *Chironomus.*

Before proceeding with a description of the results of these cytochemical experiments, some attention should be directed to an evaluation of the conditions that are essential in the application of methods employing enzymatic hydrolysis. The first of these is purity of the enzyme. In order to satisfy this requirement we have extracted, purified, and crystallized in our laboratory by the methods noted in table 1 the various enzymes used in the studies here reported. The fact that an enzyme has been crystallized by a method designed to remove contaminants does not guarantee the purity of every preparation. We anticipate the time when commercially available crystalline enzymes will carry chemical specifications as precise as those now demanded by the chemist in his purchase of inorganic chemicals. Until that time comes, however, each sample of enzyme purchased (or prepared) must be tested for interfering contaminants if unequivocal conclusions as to cytochemical specificity are to be derived from the experiments in which it is used. None of our ex-

periments has been performed without such preliminary chemical assay.

Not only must purity of the enzyme be assured, but all other variables capable of modifying the cytochemical reactions must be controlled. Some of the conditions that have been found most satisfactory for cytochemical use of the enzymes and analysis of the staining reactions are listed in table 1.

If alteration of stainability is to serve as a basis for determining the action of enzymes on cellular materials, it is

TABLE 1

ENZYME	METHOD OF PREPARATION	METHOD OF CYTOCHEMICAL USE [1]			
		Fixative	mg enzyme per ml solvent	Solvent	pH
Ribonuclease	McDonald ('48)	Acetic-alcohol	0.1–1.0	H_2O	6.0
		Flemming	1.0–6.0	H_2O	6.0
Desoxyribonuclease	Kunitz ('48, '50)	Acetic-alcohol	0.1–1.0	H_2O	6.0
		Acetic-alcohol	0.01–1.0	0.003 *M* $MgSO_4$	6.0
Pepsin	Northrop ('46)	Acetic-alcohol	0.1	0.02 *N* HCl	1.6
		Navashin	1.0–3.0	0.02 *N* HCl	1.6
		Flemming	1.0–3.0	0.02 *N* HCl	1.6

[1] Temperature, 37°C., time, 1–2 hours.

essential that the conditions under which the enzymes are used do not in themselves alter the staining reaction. The solvent in which the enzyme is dissolved must be considered, as well as the pH, time and temperature of digestion.

The pH of a solution in which tissues are immersed in the course of procedures that precede staining may alter profoundly the intensity and quality of color obtainable with basic and acidic dyes as compared with those of controls kept in distilled water (cf. Naylor, '26). For this reason the pH at which an enzyme solution is used cytochemically must be chosen after consideration of the effect of pretreatment at

this pH on subsequent staining reactions. By way of illustration: The optimum pH for the action of ribonuclease is around 7.6. It was found, however, that sections of onion root tips treated with buffers at pH 7.6, or with distilled water adjusted to this pH, stained intensely with safranine (as used in Flemming's triple stain), but could not be differentiated with the same precision as sections that had been treated at pH 6. Stainability may also be modified by the electrolytes present in the buffers that are commonly used for maintaining constant pH during enzymatic digestion (Stowell and Zorzoli, '47; Kaufmann et al., '47; Kaufmann, in press; cf. also the studies of Carter and Greenstein, '46; and Creeth, Gulland, and Jordan, '47, on the action of electrolytes on nucleic acids). Because of these modifying effects of pH and electrolytes on stainability, enzymes were used whenever possible by dissolving salt-free preparations in distilled water and adding sufficient 0.01 or 0.1 *N* sodium hydroxide to bring the pH to 6. A series of control experiments indicated that the small amounts of electrolytes required for this purpose did not perceptibly alter the staining reactions. In using either desoxyribonuclease or pepsin some special considerations are involved. Since Mg ions enhance the activity of desoxyribonuclease, magnesium sulfate was added to solutions of this enzyme. Control experiments showed that some alteration of stainability occurred after treatment with solutions of magnesium sulfate unless their concentration was very low. Concentrations greater than 0.003 *M* should therefore not be used in cytochemical studies. Pepsin is to all practical purposes inactive at pH 6, and was accordingly used dissolved in hydrochloric acid, usually 0.02 *N*, although various experiments included a range of concentrations between 0.0001 and 0.02 *N*. An evaluation of the action of pepsin in modifying stainability required consideration of the effect of the acid in these various concentrations on stainability of cellular materials.

The selection in these various experiments of conditions for enzymatic hydrolysis that in themselves do not markedly

alter staining reactions frequently required the use of the enzymes at pH levels other than those of optimum activity. In these cases high concentrations of enzyme were used, since it was desirable to complete the process of hydrolysis in a relatively short period of time. This requirement in turn stems from the observation that even water reduces basophilia when used at 25° or 37°C. over periods as long as 24 hours (or even for much shorter times at temperatures approaching or exceeding 60°C., as noted by Brachet, '40; Panijel, '47; Kaufmann, Gay, and McDonald, '49). Enzymatic hydrolysis was therefore carried out for an hour or two at 37°C. in most of our studies. When it was necessary to continue digestion over long periods of time, continuing potency of enzyme solutions was assured by constant renewal whenever the activity — as determined by chemical assay — dropped below 75% of its original value.

In addition to controlling conditions favorable for digestion and subsequent staining, it is also essential to establish and maintain rigid schedules in preparing material for digestion. It was necessary to fix the tissues in order to study the action of enzymes on the cells, since many exogenous enzymes will not penetrate the living cell, and will not act on substrate materials that are in their native state. In our studies a variety of standard cytological fixatives were used — those of Carnoy, Navashin, Bouin, Zenker, Helly, and Flemming. Since tissues immersed in such fixatives are preserved without gross distortion of the spatial relations that exist in the living cell, it may be assumed that the precipitated nucleic acids and proteins serve as valid guides to the location of these materials before fixation. Unfortunately, cytological fixatives that produce least structural deformation, such as the various modifications of Flemming, bind cellular materials so firmly that they are not easily attacked by enzymes or other chemicals. Since fixation in Carnoy's acetic acid-alcohol does not unduly inhibit enzymatic activity, it seemed expedient in these exploratory experiments to adopt this method of fixation as standard procedure, although the

coarse fixation pattern produced thereby falls far short of the cytological ideal. It will be realized, however, that even the simplest fixative, such as acetic acid, profoundly alters the reaction of cells to subsequent chemical treatment. This is illustrated in figure 1, which shows two salivary-gland cells of *Drosophila*, one fixed in acetic acid, the other unfixed, and both treated for equal periods of time in 1 *M* sodium chloride. This reagent, used by Mirsky and Pollister ('46) for extracting nucleohistone from unfixed nuclei of animal tissues, honeycombs in the span of a few hours at 0°C. the chromosomes of the unfixed salivary-gland cell (fig. 1 a), but does not distort the pattern of banding in chromosomes that have previously been fixed in 45% acetic acid (fig. 1 b). In this report we shall consider primarily fixed cells, and unless otherwise stated the fixative used was a mixture of three parts absolute alcohol and one part glacial acetic acid. We clearly recognize that results obtained in experiments on these fixed tissues can only provide a first approximation to an understanding of the chemical organization of the living cell.

Ideally, the comparison of enzyme-treated with control material should be between parts of a single fixed cell. Since this ideal can rarely be achieved, comparison should be made between parts of a single piece of tissue. In an experiment that requires treatment of fixed cells with two or more enzymes, as many as 40 or 50 preparations are needed for adequate analysis of the effects of all the variables involved. At least 50 or 60 5-μ sections can be obtained from a single onion root tip, whereas any smear technique would provide only a few preparations from a single piece of tissue. Sectioned material was therefore found to be more advantageous than smears for comparison of experimental and control treatments within one experiment. In order to ensure comparable results from one experiment to another, it was necessary to eliminate differences in material due to technical treatment. Rigid schedules were maintained for dehydration, clearing, and imbedding, care being taken during the last process to avoid

exposure of material to elevated temperature (above 60°C., cf. Kurnick, '50a).

In order to use minimum quantities of enzymes, paraffin sections were mounted on cover slips that could be fitted into small containers during the period of enzymatic hydrolysis. After digestion, experimental and control materials for any single experiment were rinsed thoroughly in distilled water, and placed together in racks that had been designed to carry large numbers of the glass slips. All sections were thus transferred simultaneously to the dish containing the stain. After staining, the sections were again rinsed thoroughly to insure removal of dye that was not firmly attached.

These requirements concerning purity of enzyme and control of experimental variables having been fulfilled, there seems a substantial basis for accepting methods employing enzymatic hydrolysis as valid cytochemical procedures. Experiments formulated on this premise have confirmed results obtained by other cytochemical and chemical methods, and have furnished additional information with respect to the in situ location and patterns of association of cellular materials during the various phases of mitosis. As has been said elsewhere (Kaufmann, Gay, and McDonald, '49), this statement is not meant to imply that the method of enzymatic hydrolysis is self-sufficient, since it should be clearly recognized that each of the cytochemical and chemical procedures has its limitations and advantages with respect to identification and location of cellular components. For example, as will be illustrated later, the possibility must be constantly considered that the specific removal of one material by an enzyme may result in the nonspecific loss of other materials.

Our cytochemical studies were designed with three objectives in view: (1) recognition and location, (2) determination of patterns of association, (3) quantification. Some aspects of studies directed to the first two of these objectives are considered briefly in this report. Complexities of analysis

encountered at these levels have so far prevented a concerted effort on our part to attain the third objective.

IDENTIFICATION AND LOCATION OF NUCLEIC ACIDS

Identification of nucleic acids by coloration with suitable dyes represents a widespread cytochemical practice. The Feulgen reaction is generally recognized as a specific chemical test for desoxyribonucleic acid (DNA). The DNA identified thereby is restricted to chromatin, and is readily removed by desoxyribonuclease even in low concentrations (fig. 2). The routine staining reactions for ribonucleic acid (RNA) involve basic dyes. Brachet ('40) used pyronine for identifying RNA by staining cells with the Unna-Pappenheim combination of methyl green and pyronine. Toluidine blue, methylene blue, and other basic dyes have also been used to color RNA; we have found safranine especially serviceable (Kaufmann, McDonald, and Gay, '48). Without imputing chemical specificity to these dyes, it is possible to manipulate staining procedures so that RNA is selectively colored, as indicated by impairment of stainability after treatment with purified ribonuclease. In experiments using these methods RNA has been shown to occur in cytosome, spindle, nucleoli, and chromosomes (Kaufmann, McDonald, and Gay, '48; Kaufmann, Gay, and McDonald, '49).

That RNA is present in chromosomes had been suggested by Brachet ('40) and Schultz ('41) on the basis of altered stainability effected by treatment with ribonuclease; but these experiments were open to question because of the lack of chemical specificity of the dyes used and the possible presence of proteolytic contaminants in the samples of ribonuclease then available. Three years ago attention was again focused on the RNA content of chromosomes by Mirsky and Ris ('47b) in a chemical analysis of extracted chromosomes, and by our finding that proteolytic-free ribonuclease degrades a pyronine-staining chromosomal component during all phases of mitosis (reported in the discussion of papers by Brachet and Mirsky at the 1947 Cold Spring Harbor Symposium).

Our cytochemical analysis thus confirmed Brachet's tentative conclusions, and the use of safranine extended the concept of the functional relations of chromosomal RNA. It is well known that the safranine and gentian violet combination of dyes, when used after fixatives containing chromic, osmic, and acetic acids, permits a pattern of differentiation in meristematic cells of root tips in which metaphase and anaphase chromosomes are bright red whereas the chromonematic threads of late telophases, early prophases, and interphases are violet. Ribonuclease attacks this red-staining component, so that the condensed chromosomes appear violet after digestion. We have observed this reaction in various types of cells fixed in Flemming's fluid — for example, in meristematic cells of onion roots and spermatogenous cells of grasshopper testes (fig. 3). These observations suggest that the RNA of condensed chromosomes is present in a form different from that of chromosomes at other stages. It seems possible that a cycle of accretion and dispersion of RNA occurs in the course of mitosis coincident with the disappearance and reappearance of the nucleolus, and that the incorporation of this RNA in the chromosome is related to the control of genic activity (Kaufmann, McDonald, and Gay, '48).

The question arises whether the loss of stainability with safranine effected by ribonuclease on Flemming-fixed cells is related to the high concentration of ribonuclease required to degrade RNA after such fixation (a 0.6% solution was used in our experiments). It seemed desirable to determine, therefore, whether differential staining of condensed chromosomes could be obtained in acetic-alcohol-fixed cells, which are more easily degraded by ribonuclease. It was indeed possible to secure such differentiation after staining with safranine (even without the customary supplementary treatment with gentian violet) if sections of Carnoy-fixed root tips were immersed for a minute in a 0.1 *N* solution of hydrochloric acid and then rinsed thoroughly in distilled water before being placed in the dye. Under these conditions the condensed chromosomes of the undigested cells color bright

red, and those of the resting stages a pale purple, whereas the condensed chromosomes of the digested cells (one hour in a 0.01% solution of ribonuclease) show the same pale purple color as the chromatin of the resting stages. Alteration of a safranine-stainable component of condensed chromosomes by low concentrations of ribonuclease lends support to the contention that these chromosomes contain RNA which is either of a different type or is bound differently from that of the chromosomes of the resting stages. Other experiments indicate that safranine is staining both ribo- and desoxyribonucleic acids, since stainability is impaired by digestion with either ribonuclease or desoxyribonuclease. The pattern of association of these two types of nucleic acid in the condensed chromosome will be discussed subsequently.

INTERRELATIONS OF NUCLEIC ACIDS AND PROTEINS

On the basis of the foregoing observations it is apparent that digestion of fixed cells with desoxyribonuclease or ribonuclease, and staining with suitable dyes, serve to identify and locate DNA or RNA. The further question then remains whether enzymatic action will also serve to reveal patterns of their association.

Chemical extractions of animal nuclei have tended to emphasize two separable nucleoprotein complexes, a RNA-tryptophane protein, and a DNA-histone (Mirsky and Ris, '47b). The presence of ribonucleoprotein in fixed cells can be demonstrated cytochemically by treating sections with ribonuclease and staining with acidic dyes such as fast green, light green, and acid fuchsine. The action of the enzyme increases stainability of chromosomes, nucleoli, and cytoplasm with these dyes (figs. 4 a, b). This result has been attributed to the action of the enzyme in degrading ribonucleoprotein so as to release new sites for attachment of the dye molecules (Kaufmann, McDonald, and Gay, '48). Stainability can be inhibited by using proteolytic enzymes such as pepsin and trypsin after ribonuclease (figs. 4 c, d). This suggests that the material staining with acidic dyes is protein. Its dis-

tribution in the cell roughly parallels that of the blue color obtained in cytochemical application of the May-Rose test for tryptophane, indicating that at least some of the protein liberated by the action of ribonuclease contains tryptophane. Unfortunately there is no precise cytochemical test for histone. The report of Mazia, Hayashi, and Yudowitch ('47) that pepsin degrades more acidic proteins but not histone or nucleohistone seemed to offer a possible cytochemical basis for discriminating between these two types of proteins. Since that report was based on a study of both soluble and fibrous protein substrates, we attempted to confirm these observations by preparing and fixing in acetic-alcohol spots and "fibers" (actually folded films obtained by spreading the protein on the surface of an appropriate buffer and compressing between waxed barriers) of egg albumin (crystallized by the method of La Rosa, '27), and of histone and nucleohistone (extracted from thymus-gland cells by the method of Mirsky and Pollister, '46, and Mirsky and Ris, '47a, b). Spots and "fibers" of egg albumin were rapidly digested by pepsin, confirming the statement of Mazia et al., that pepsin "digests fibers" of "more acidic" proteins. We were unable, however, to confirm the report of these workers that pepsin fails to digest histone, because the spots and "fibers" that we had prepared disintegrated rapidly when treated with low concentrations of the enzyme. On the basis of these results it is apparent that pepsin could be expected to digest both histone and more acidic proteins in tissue sections.

A useful cytochemical procedure was uncovered in these experiments, however, by our observation that treatment with 0.02 *N* hydrochloric acid for two hours at 37°C. (or with 1 *N* hydrochloric acid for 10 minutes at 60°C.) impairs stainability with acidic dyes of the histone component of the desoxyribonucleohistone spots and "fibers," but does not impair stainability of egg albumin spots and "fibers." It might be expected, therefore, that if histone were released from ribonucleoprotein by the action of ribonuclease on tissue sections, subsequent treatment with 0.02 *N* hydrochloric

acid would impair stainability with acidic dyes. When sections of onion root tips that had been fixed in acetic-alcohol were treated with ribonuclease and then stained with fast green, a bright green color was obtained in cytoplasm, nucleoli, and chromosomes; but if the cells were treated first with ribonuclease, and then with 0.02 *N* hydrochloric acid, bright green color appeared in cytoplasm and nucleoli, but not in the chromosomes (fig. 5). If treatment with ribonuclease, however, was followed by treatment with pepsin, cytoplasm and nucleoli as well as chromosomes were devoid of green color. Details of these studies are reported elsewhere (Kaufmann, Gay, and McDonald, in press). The results of this series of experiments suggest that the material in the chromosomes that stains with acidic dyes after treatment with ribonuclease is probably of the histone type, whereas the protein of cytoplasm and nucleoli is rich in tryptophane. In other experiments on squashes of salivary-gland chromosomes of *Drosophila melanogaster* fixed in 45% acetic acid it was found that 10-minute hydrolysis in 1 *N* hydrochloric acid at 60°C. (incident to application of the Feulgen technique) after treatment with ribonuclease did not inhibit subsequent stainability of the band or interband regions with fast green. It seems probable that in this case chromosomal RNA is associated with a protein rich in tryptophane.

Thus, by using ribonuclease in combination with a dye that is not staining RNA but is coloring protein, it has been possible to demonstrate cytochemically the association of RNA with protein. It has also been possible to demonstrate cytochemically the association of DNA with protein. When sections of onion root tip are digested with desoxyribonuclease and stained with fast green there is an increase in the amount of dye combined in the chromosomes as compared with the amount in the undigested controls (figs. 6 a, b). Treatment with 0.02 *N* hydrochloric acid after treatment with desoxyribonuclease only partially impairs stainability of the chromosomes with this acidic dye (fig. 6 c). Stainability is markedly reduced, however, by aqueous trypsin. Treatment

with pepsin after desoxyribonuclease results in complete disintegration of the nucleus (fig. 6 d). This series of observations suggests that the action of desoxyribonuclease on chromosomal DNA releases a nonhistone protein in addition to histone.

Methods of enzymatic hydrolysis have also furnished evidence that RNA and DNA are intimately associated in the fixed chromosome. The first line of evidence was obtained by digesting cells with ribonuclease and then staining to locate DNA. The Feulgen colorability of chromatin is unaffected by ribonuclease digestion (fig. 7). On the other hand, methyl green, which stains highly polymerized DNA (Kurnick, '50b; Pollister and Leuchtenberger, '49), will color undigested chromosomes intensely, whereas no color is detectable in chromosomes after treatment with high concentrations of ribonuclease (fig. 8). This might be interpreted as indicating that chromosomes contain highly polymerized RNA, which stains with methyl green, and is depolymerized by ribonuclease. However, such action would not account for the lack of stainability with methyl green of the highly polymerized DNA that presumably would remain in these chromosomes. Moreover, desoxyribonuclease also reduces methyl green stainability of chromosomes (fig. 9); but at the same time it inhibits Feulgen stainability (cf. fig. 2). The action of ribonuclease in impairing stainability with methyl green raises the question whether this enzyme contains a desoxyribonuclease contaminant. The answer was furnished in part by the failure of ribonuclease to reduce Feulgen stainability. Chemical assay also indicates that the ribonuclease which we have crystallized and purified contains no appreciable amount of desoxyribonuclease. One part of desoxyribonuclease in 20,000 parts of ribonuclease is detectable in these tests, so that any possible desoxyribonuclease contaminant must be less than this amount. Concentrations of desoxyribonuclease of this order of magnitude when used under the same experimental conditions as were employed in the ribonuclease tests did not impair methyl-green stainability of chromosomes. These

results thus indicate that failure to stain with methyl green after treatment with ribonuclease is not due to the action of a desoxyribonuclease contaminant.

The possibility that ribonuclease per se can in addition to degrading RNA also degrade DNA has already been negated by the numerous observations of other workers (e.g., see Jones, '20; Dubos, '37; Kunitz, '40). This possibility is also nullified in part by our qualitative and quantitative studies of the effect of ribonuclease in low and high concentrations on Feulgen stainability of chromosomes. Ribonuclease used in concentrations as high as 0.6% over a period of 24 hours did not reduce Feulgen stainability of chromosomes of onion root tip or amphibian liver, as determined both by visual observation and by preliminary application of the spectrophotometric technique of Pollister and Ris ('47). We have also tested repeatedly during the past year the action of ribonuclease on dried and fixed drops of polymerized and depolymerized DNA prepared from isolated cell constituents, and on "fibers" of desoxyribonucleohistone. In all these tests we have found no evidence that ribonuclease degrades either polymerized or depolymerized DNA.

Because our experimental evidence indicates that contamination of enzyme preparations or nonspecificity of their action cannot account for the similarity of ribonuclease and desoxyribonuclease in inhibiting the stainability of chromosomes with methyl green, we suggest that this results from a modification in the pattern of alignment of those chromosomal materials that remain after one or the other of the nucleic acids has been degraded. Degradation of one material might in some cases result in the actual loss from the chromosome of an associated material. Such loss, however, does not seem to occur when ribonuclease is used, for this enzyme does not impair Feulgen stainability even though it inhibits stainability with methyl green. The explanation of pattern realignment is merely an extension of our earlier proposal that the chromosome is an integrated fabric in which no single nucleic acid or protein can be regarded as the primary structural

component (Kaufmann, '49; Kaufmann, Gay, and McDonald, '49). It seems possible that chromosomes may contain a mixed nucleic acid composed of both desoxyribose and ribose nucleotides. If the ribonucleotides occupy intercalary positions and the number of adjacent desoxyribonucleotides is large, removal of the ribose nucleotides by the action of ribonuclease would effectively depolymerize the complex nucleic acid without, however, impairing its Feulgen colorability. This type of structure would thus account for the decrease in methyl-green stainability without loss of Feulgen stainability when sections are digested with ribonuclease, and for the decrease in methyl-green stainability concurrently with Feulgen stainability when sections are digested with desoxyribonuclease.

Complex patterns of organization of chromosomal materials have also been revealed in experiments using proteolytic enzymes (Gay, '49); but these data will not be presented in this report. We should like, however, to direct attention briefly to the use of trichloroacetic acid (TCA) for the removal of nucleic acids in cytochemical studies. Treatment of acetic-alcohol-fixed tissue sections with TCA at 90°C. for 10 to 15 minutes removes both RNA and DNA. This results in inhibition of coloration with the Feulgen method and loss of stainability with basic dyes. On the other hand, stainability with acidic dyes is enhanced markedly as compared with that of controls treated with TCA at 0°C. (fig. 10). This increase in stainability is again referable to degradation of nucleoproteins. Similar results can be obtained by using both ribonuclease and desoxyribonuclease in succession. Treatment of cells with pepsin after hot TCA or after treatment with the two nucleases results in cellular disintegration. With respect to the chromosomes it should be pointed out that removal of both nucleic acids does not efface these bodies (Kaufmann '49). The subsequent action of pepsin in hydrochloric acid is sufficient to remove histones and to degrade the tryptophane proteins. The chromosome is then obliterated.

We have attempted in this short survey to indicate the validity of cytochemical methods employing enzymatic hydrolysis. A long series of experiments some of which have been reported here, with many replications of the more critical ones, has convinced us that the necessary safeguards in the application of these cytochemical methods to the analysis of biological problems can only be attained by close coordination of cytological and biochemical techniques. Methods employing enzymatic hydrolysis can then be applied to the analysis of more complex cellular substrates, such as are found in the chromosomes.

Our experimental results have focused attention on the intricate patterns of association of cellular materials in fixed tissues, and the cyclic changes of these materials in the course of mitosis. The acetic-alcohol fixative used in many of these experiments is not wholly satisfactory; progress must be made in digesting cells preserved more adequately. We must also not forget in our analysis of the experimental data that the living cell is not merely a finely balanced system of nucleic acids and proteins. For these and other obvious reasons we do not pretend that we have gone far in delineating "in the exiguous confines of the cell," to quote Professor R. R. Bensley (in Glick, '49), "that elusive and mysterious chemical pattern which is the basis of life." But we do submit the contention that methods involving enzymatic hydrolysis represent an important experimental procedure in the approach to this objective.

SUMMARY

The use of purified enzymes in combination with various staining procedures affords a reliable cytochemical method for identifying nucleic acids in fixed tissue sections and determining their patterns of association with proteins. The validity of interpretations of cellular organization derived from the application of these methods depends on the adequacy with which the experimental variables are controlled.

By treating sections with ribonuclease and staining with basic dyes such as pyronine and safranine, it is possible to identify ribonucleic acid. By treating with this enzyme and staining with acidic dyes, it has been determined that ribonucleic acid is combined in the fixed cell with protein. By treating with ribonuclease and then with dilute hydrochloric acid before staining with acidic dyes, we have found evidence that the protein moiety of the ribonucleoprotein in the chromosomes is probably of the histone type, whereas the protein of cytoplasm and nucleolus is a more acidic type, rich in tryptophane.

Treatment of cells with ribonuclease does not reduce stainability of chromosomes with the Feulgen reagent, but does destroy stainability with methyl green. These results suggest that the chromosomes contain a nucleic acid composed of both ribose and desoxyribose nucleotides.

Desoxyribonuclease effaces stainability of fixed cells with the Feulgen reagent and thereby serves to locate chromosomal desoxyribonucleic acid. The results of treating with this enzyme and then with dilute hydrochloric acid before staining with acidic dyes indicate that desoxyribonuclease releases from chromosomal desoxyribonucleoprotein a non-histone protein in addition to histone.

Cytochemical methods employing enzymatic hydrolysis are thus shown to be especially serviceable in revealing the complex patterns of association of cellular materials.

OPEN DISCUSSION

Chairman Mazia: Dr. Kaufmann's paper is before you for discussion.

Dr. Pollister: Although he did not say so I suppose the reason that Dr. Kaufmann talked so much about whether ribonuclease could degrade DNA was partly due to the trouble I got into a short time ago. This is due, perhaps, to the fact that when we use enzymes we do not enjoy the advantages of close collaboration with Dr. McDonald. The results that we have obtained, however, with a sample of ribonuclease

obtained from Armour and Co., and further purified by boiling in magnesium sulfate solution raise some questions. We were attempting to disentangle the two nucleic acids by using ultraviolet absorption measurements in conjunction with ribonuclease. We digested large liver nuclei, of which Dr. Kaufmann has just shown you a sample, with ribonuclease in a concentration of 2 mg per milliliter and followed the three separate components of the nucleotides as follows: desoxypentose by the Feulgen reaction, phosphoric acid with azure A staining, and total purines and pyrimidines by their natural ultraviolet absorption, with correction by a measured blank. We obtained these results based on very extensive data from hundreds of nuclei. To our surprise all three components were reduced to the same extent. Some nuclei were followed by first measuring the ultraviolet absorption and then doing the Feulgen on exactly the same nuclei. From this kind of result you cannot follow pentose directly. Either all the polynucleotides that were removed were desoxypolynucleotides or else the pentose and desoxypentose polynucleotides were removed at the same rate. Either ribonuclease was digesting DNA or our ribonuclease was contaminated with desoxyribonuclease. If the latter is so it seems to me that the contaminant must be a somewhat different desoxyribonuclease from the usual one, since it was heat stable. Dr. Moses has obtained similar results with one of Worthington's preparations, but to a less marked extent. It is apparent from what Dr. Kaufmann has told us this morning, that if you have a preparation of ribonuclease in which you have determined that there is no desoxyribonuclease activity, then it will not affect the Feulgen reaction and you are in the happy state where you can go ahead and really find out how much of the natural ultraviolet absorption is due to ribonucleotides and how much to desoxyribonucleotides.

Chairman MAZIA: We know from Barton's work in our laboratory that there are at least two desoxyribonucleases and that one is a contaminant of the other in Kunitz's preparation. His method effects a tremendous concentration of the

viscosity-lowering enzyme but leaves a small contamination of the enzyme measured by the protamine method. One of them would affect the methyl green staining without affecting the Feulgen. The combination, which you get in crystalline desoxyribonuclease, would affect both. Of course we have no idea as to whether the one that affects just the methyl green could exist as a contaminant unless Dr. McDonald has checked that with Barton's recently published method.

Dr. Kaufmann: Dr. McDonald can answer specifically concerning methods of assay, but I should like to re-emphasize that we are dealing here with ribonuclease, and that the preparation crystallized and purified in our laboratory did not reveal any detectable trace of desoxyribonuclease. The solution of ribonuclease used by Dr. Pollister has been shown cytochemically to have properties different from the solution used in our studies. The basis for the differences remains to be determined.

Dr. McDonald: I have not used Barton's protamine method in assaying our ribonuclease. It has been assayed chemically by the viscosimetric technique, by Kunitz's spectrophotometric technique, and by the rate of formation of acid-soluble split products. It has also been tested cytochemically for its ability to change the stainability with methyl green of spots or films of isolated DNA or desoxyribonucleohistone. All such assays have been negative. Depolymerization by heat of desoxyribonucleic acid following the techniques of Kurnick results in loss of viscosity and ability to stain with methyl green without markedly altering its acid precipitability. It is my understanding that, of the two enzymes mentioned by Dr. Mazia, the one which might alter the capacity of DNA to stain with methyl green without altering its ability to react with the Feulgen reagent is the viscosity-lowering enzyme. The samples of ribonuclease used in these experiments contained less than one part in 50,000 of this enzyme, a concentration which in our experience does not reduce methyl green or Feulgen stainability.

As far as Dr. Pollister's data are concerned, several explanations based on the assumption that his ribonuclease was contaminated with desoxyribonuclease appear probable. In the first place there may be, in comparison with the amount of DNA, very little RNA in chromosomes. The decrease in ultraviolet absorption and azure-A staining due to digestion of RNA may therefore be a small percentage of the total decrease. One must also be rather cautious, I feel, in using ultraviolet absorption methods to measure quantitatively the amounts of purines and pyrimidines present in complex compounds since the ultraviolet absorption of such compounds depends not only on the total amount of purines and pyrimidines present but also on the configuration of the compound. Kunitz, for example, has shown that the ultraviolet absorption of DNA increases markedly during digestion with desoxyribonuclease, the ultimate increase being nearly 30%. It is therefore conceivable that the DNA remaining in Dr. Pollister's sections after digestion with desoxyribonuclease-contaminated ribonuclease might have a higher specific absorption than the original DNA, so that the percentage drop in the ultraviolet absorption may not give a true picture of the amount of nucleic acids that has been removed from the sections.

Dr. Pollister: Ribonuclease does not seem to remove this azure stain that we regard as being so highly specific for polynucleotides. Ribonuclease in low concentrations does not appear to affect chromosomes, indicating either that there is not much ribonuclease in most chromosomes or that, for some reason, it is not easily removed. I think from the analytical results which people are getting that there is probably but a small amount of RNA in chromosomes.

I thought possibly that here we were showing the complex that Dr. Kaufmann was talking about and that, while ribonuclease can degrade the pentose nucleic acid, the whole complex can not get out of the nucleus until the DNA is broken down.

Dr. McDonald: The large amounts of DNA present in chromosomes may affect the rate of digestion by ribonuclease of RNA. We must also remember that there may be many different ribonucleic acids which are probably hydrolyzed at quite different rates. Dr. Schmidt might like to comment on this point.

Dr. Schmidt: We find that RNA isolated from pancreas is not attacked by Kunitz's ribonuclease. Dr. Kerr has had the same experience. Therefore, I would be very much interested in knowing how sections of pancreas behave on treatment with ribonuclease.

Dr. Kaufmann: We have not tested the action of our ribonuclease on pancreas.

Chairman Mazia: Do you have any theory as to why these depolymerizing enzymes should be so effective in removing nucleotides so completely when presumably they are attached to proteins anyway?

Dr. Schmidt: The experience one has when working with ribonuclease is that it takes very little action for the enzyme to profoundly alter the solubility of yeast nucleic acid in dilute solutions — in fact so little that mononucleotide formation might not even be, chemically, easily detectible.

Then another trouble occurs to me which I have not studied quantitatively. It seems to me that the amount of secondary phosphoryl groups is very important for the formation of insoluble nucleoprotein complexes — more important than the cross-linkages.

Dr. Mirsky: I missed a point in your talk. What happens when you take isolated DNA that stains with methyl green and then treat it with this ribonuclease?

Dr. McDonald: The capacity for isolated DNA to stain with methyl green is not reduced by treatment with ribonuclease. Spots of isolated DNA were shown on one of the slides. We have also used spots and fibers of isolated desoxyribonucleohistone. In no case was the methyl-green stainability reduced by ribonuclease.

Dr. Kaufmann: I am wondering if Dr. Mirsky was inquiring about the action of ribonuclease on material that had been stained in methyl green. In all the cases I reported the staining was done after treatment with the enzymes. There are a few dyes that one can use first and still get enzymatic digestion, but most of them will block the action of the enzyme.

Dr. Mirsky: These remarks are very interesting. Some people think that DNA is not as highly polymerized in the chromosomes as it is when we prepare it. Your results may be an indication of just that. Maybe it is highly polymerized with just the RNA in it. Suppose you take some cells and treat them with ribonuclease so that they no longer stain with methyl green, and now isolate from these cells, DNA. Will that then combine with methyl green? If you did this experiment and got out a polymerized DNA that would react with methyl green, you would have very good evidence that DNA really has been polymerized in the course of the preparation. It is quite possible.

Dr. McDonald: The experiment you mentioned has not been tried and might be rather difficult to perform. If one digested with ribonuclease without fixing the cells there would undoubtedly be a great deal of autolysis during the digestion period. If one fixes them before digestion then the usual methods of extraction of the DNA are unfeasible. It is, however, an experiment well worth thinking about.

Dr. Hotchkiss: Concerning Dr. McDonald's remarks about Dr. Pollister's slide, if I understand correctly, it seems to me that the situation would be this: while it is quite true that in solution you would have a rise in the specific absorption upon digestion with desoxyribonuclease, in the cytochemical studies the split products of digestion would be removed and would not be measured.

Dr. McDonald: In Dr. Pollister's work all the DNA has been depolymerized so that it no longer stains with methyl green. However, there are still present some particles of DNA which, although depolymerized, give the Feulgen reaction. It is these particles that are giving the ultraviolet

absorption and which may have a different specific absorption from the nondepolymerized form.

DR. POLLISTER: I am afraid that we may be talking at cross purposes. I did not mean to give the impression, when I showed those three percentage reductions in extinction, that I can equate the desoxypentose to the nucleotides and to the phosphoric acid. What we are demonstrating is the removal of a certain proportion of the phosphoric acid groups, a certain proportion of the desoxypentose, and a certain proportion of the ultraviolet absorption.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

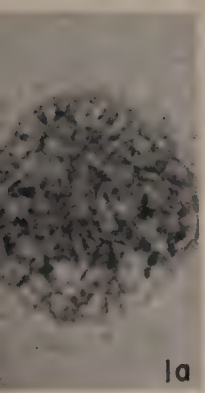
1 Salivary-gland cells of *Drosophila melanogaster*. (a) Dissected in Ringer's solution and then treated with 1 *M* sodium chloride at 0°C. for 12 hours. (b) Dissected in Ringer's solution and fixed in 45% acetic acid before treating with 1 *M* sodium chloride at 0°C. for 12 hours.

2 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol solution and stained by the Feulgen reaction. (a) $MgSO_4$ Control — immersed in 0.003 *M* $MgSO_4$, pH 6, for 1 hour at 37°C. (b) Desoxyribonuclease-treated — 0.1 mg per milliliter of desoxyribonuclease in 0.003 *M* $MgSO_4$ adjusted to pH 6 — 1 hour, 37°C.

3 Five-micra sections of grasshopper testis fixed in Flemming's fluid and stained with safranine, gentian violet and orange G. (a) Water control — immersed in distilled water for 2 hours at 37°C. (b) Ribonuclease-treated — 6 mg per milliliter of ribonuclease in water adjusted to pH 6 — 2 hours, 37°C.

4 Five-micra paraffin sections of onion root tip fixed in Flemming's fluid and stained with fast green. (a) Water control — immersed in distilled water, pH 6, for 2 hours at 37°C. (b) Ribonuclease-treated — 6 mg per milliliter of ribonuclease in water adjusted to pH 6 — 2 hours, 37°C. (c) Ribonuclease followed by 0.02 *N* HCl — ribonuclease treatment as above followed by 0.02 *N* HCl for 2 hours at 37°C. (d) Ribonuclease followed by pepsin — ribonuclease treatment as above followed by pepsin — 3 mg per milliliter in 0.02 *N* HCl — 2 hours, 37°C.

5 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol and stained in fast green. (a) Ribonuclease treated — 1 mg per milliliter of ribonuclease in distilled water adjusted to pH 6 — 2 hours, 37°C. (b) Ribonuclease treatment followed by 0.02 *N* HCl for 2 hours at 37°C.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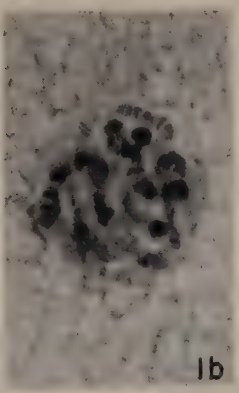
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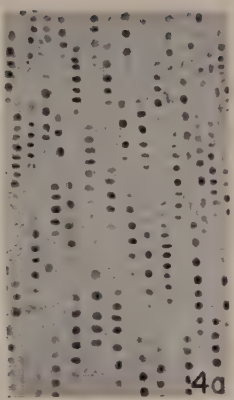
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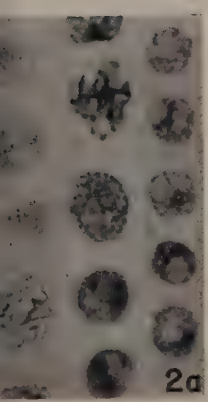
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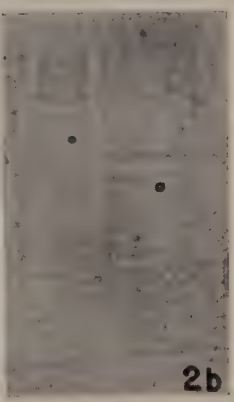
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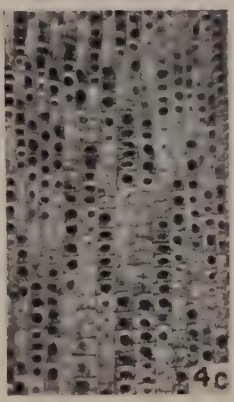
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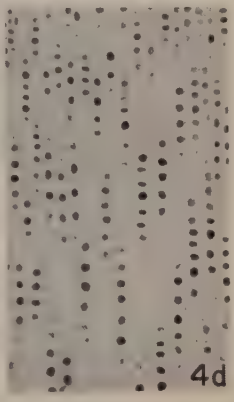
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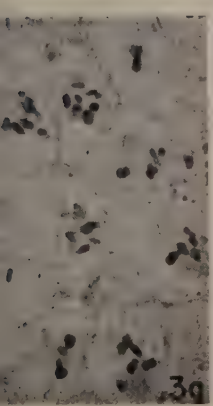
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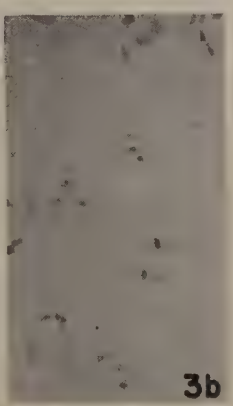
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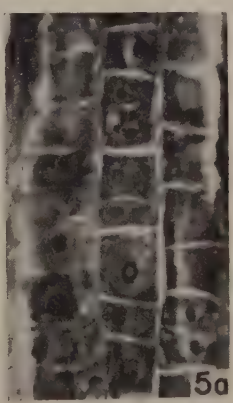
5a


5b

PLATE 2

EXPLANATION OF FIGURES

6 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol and stained in fast green. (a) 0.003 *M* $MgSO_4$ control — section treated in 0.003 *M* $MgSO_4$ adjusted to pH 6 — 1 hour, 37°C. (b) Desoxyribonuclease-treated — 1 mg per milliliter of desoxyribonuclease in 0.003 *M* $MgSO_4$ adjusted to pH 6 — 1 hour, 37°C. (c) Desoxyribonuclease treatment followed by 0.02 N HCl — desoxyribonuclease treatment as above followed by 0.02 *N* HCl for 2 hours at 37°C. (d) Desoxyribonuclease treatment followed by pepsin — desoxyribonuclease treatment as above followed by pepsin — 3 mg per milliliter, in 0.02 *N* HCl — 2 hours, 37°C.

7 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol and stained by the Feulgen reaction. (a) Water control — treated in water, pH 6, 2 hours, 37°C. (b) Ribonuclease-treated — 6 mg per milliliter of ribonuclease in water adjusted to pH 6 — 2 hours, 37°C.

8 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol and stained with methyl green. (a) Water control — treated in water, pH 6 — 2 hours, 37°C. (b) Ribonuclease treated — 6 mg per milliliter of ribonuclease in water adjusted to pH 6 — 2 hours, 37°C.

9 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol and stained with methyl green. (a) 0.003 *M* $MgSO_4$ control — immersed in 0.003 *M* $MgSO_4$ adjusted to pH 6 — 1 hour, 37°C. (b) Desoxyribonuclease treatment — 0.1 mg per milliliter of desoxyribonuclease in 0.003 *M* $MgSO_4$ adjusted to pH 6 — 1 hour, 37°C.

10 Five-micra paraffin sections of onion root tip fixed in acetic-alcohol, stained in fast green. (a) 0.3 *N* trichloroacetic acid (TCA), 0°C., 15 minutes. (b) 0.3 *N* TCA 90°C., 15 minutes.


6a
6b
8a
8b
6c
6d
9a
9b
7a
7b
10a
10b

STUDIES ON THE DESOXYPENTOSE NUCLEIC ACID CONTENT OF ANIMAL NUCLEI

ARTHUR W. POLLISTER, HEWSON SWIFT, AND MAX ALFERT

Columbia University, University of Chicago and University of California, Berkeley

FOUR FIGURES

INTRODUCTION

Nucleic acids bulk so large in the composition of cells that it seems certain that these substances are involved in some important biological functions. Nevertheless, with one exception, there has been no proof of a specific biological function mediated by one of these polynucleotides; no enzyme, hormone, vitamin, or even vague "growth substance" has been found to be a nucleic acid. The highly significant exception is that desoxyribonucleic acid (DNA) has been demonstrated (Avery, MacLeod, and McCarty, '44; Boivin et al., '45) to be the bacterial transforming principle; that is, the DNA extracted from one strain of bacteria permanently alters a related strain to resemble that from which the DNA was derived. This phenomenon, which has been called directed mutation, suggested to these workers that DNA must certainly be very close to the actual genic material; and that, therefore, the concept of a gene as nucleoprotein must be revised to state that at least part of the specificity is probably due to DNA rather than to protein. This conviction apparently led Boivin, in what were to be the final researches of his life, to undertake quite different confirmatory researches. He must have asked himself, "If DNA is the genic material, what are the most firmly established facts about genic behavior with which one should be able to correlate the desoxyribonucleic acids?" Obviously the best founded fact is that

the distribution of the genes in cell reproduction and biparental inheritance is completely parallel with that of the chromosomes. Therefore, if the gene is DNA its distribution should run parallel with the chromosome and gene cycles. But in bacteria the knowledge of both chromosomes and genes is incomplete; it is only in higher forms that these correlations have been well established. So Boivin and his collaborators, the Vendrelys (Boivin, Vendrely, and Vendrely, '48), turned from their field of microbiology, to study the DNA content of nuclei of mammalian tissues. They isolated masses of nuclei from various beef organs, by counting an aliquot estimated the total number of nuclei in the mass, by analysis determined the amount of DNA in the mass, and by simple division found the amount of DNA per single nucleus (table 1). The amount

TABLE 1

DNA per nucleus, beef tissues

ORGAN [1]	AMOUNTS [1] ($\times 10^{-9}$ mg)	NUMBER OF NUCLEAR TYPES	PROBABLE CHROMOSOME NUMBER
Thymus	6.4	1	diploid
Liver	6.4	2	diploid
Pancreas	6.9	6	diploid
Kidney	5.9	10	diploid
Sperm suspension	3.3	1	haploid

[1] After data of Vendrely and Vendrely ('48).

was very nearly the same for nuclei from the different organs, confirming the expectations from the fact that all these are diploid nuclei and presumably all have the same genic and chromosomal complement. The results are really more significant than they may seem at first glance, for these 4 analyses must represent means of nearly 20 different nuclear types (column 3, table 1); and the similarity of the different averages is indeed strong presumptive, though not completely conclusive, evidence that the specialization of a somatic tissue nucleus in one direction or another does not greatly change the DNA content. This amount of DNA which was character-

istic of diploid beef nuclei, was also compared with that in the haploid nuclei of bull sperm and it was found that each sperm nucleus contained approximately half as much DNA as the somatic tissue cells. The amount of DNA thus appeared to parallel strictly the distribution of chromosomes and genes, as predicted from the experiments on bacteria. Vendrely and Vendrely ('48, '49, '50) have reported similar results with several other mammals and some lower vertebrates. Mirsky and Ris ('49) have reported that the nuclei of blood and liver of a number of lower vertebrates contain twice as much DNA as does the sperm. In mammals, contrary to Boivin and Vendrely, they found that the amount per diploid nucleus of beef tissues was significantly more than twice that of the sperm. Mirsky and Ris have stated that this is too great to be accounted for by the amount of polyploidy in beef organs, and suggest that, "some other duplication of genic material is responsible for the analytical results."

It is possible to approach the question of the DNA content of nuclei by the cytological method of photometric analysis of individual nuclei, and it seemed useful to undertake this because from it should emerge a much sharper picture of the relationship of DNA to chromosomes throughout the whole life of the organism. (a) This would show conclusively whether all diploid nuclei do indeed have the same amount or whether the Boivin and Vendrely values are averages of nuclei of varying DNA content, which happen to equal twice that of the sperm nucleus. (b) It should also be possible to determine whether there is any special mode of "duplication of genic material." (c) It was also hoped to clear up some cytological findings which, if true, were quite irreconcilable with Boivin's views; for example that DNA was said to disappear entirely from oöcyte nuclei, and to vary greatly in amount during different cleavage stages of the egg.

METHODS

The process of fixation of tissue leaves in the cell what is essentially little more than a nucleoprotein mass, containing

both ribo- and desoxyribonucleic acids. One can make a cytological photometric analysis for DNA quite without interference from the ribonucleic acid (RNA) by taking advantage of the remarkably specific Feulgen nucleal reaction for desoxypentose. (For further details of technique see Swift, '50.) In practice, tissues are cut so that many whole nuclei are in the sections; whole nuclei that are spherical, or nearly so, are selected; their size is determined; and then the absorption of a central cylindrical core or plug through the nucleus is measured, using the apparatus shown in figure 1. The raw data are the transmissions of these plugs. To get an estimate of the total dye in the nucleus (and hence total DNA) these raw data must be evaluated in terms of the nuclear size. Such an arbitrary figure, with no particular absolute significance, is obtained by multiplying the extinction (E) (logarithm of the reciprocal of the transmission) by the square of the core radius (r), and dividing this product by the fraction (F) of the whole nucleus which the measured core represents. Thus, in tables 2–5, the arbitrary DNA (or Feulgen) values represent Er^2/F.

In all cytological photometry it is found that the individual measurements vary over a considerable range, within which they form a fairly symmetrical unimodal distribution curve (Pollister and Swift, '50). The cytological techniques which are useful for quantitative photometric studies are those in which these means are more or less reproducible. With carefully standardized procedure the Feulgen reaction, employed in the studies to be reported below, results in slides in which the means are reproducible to within 10% of the total optical density (Pollister, '50; Leuchtenberger, Vendrely, and Vendrely, '51). The validity of the Feulgen reaction for determination of relative amounts of DNA has been indicated by Ris and Mirsky's ('49) comparison of results obtained on cytological preparations with relative amounts of DNA determined in the same material by the Boivin-Vendrely technique.

RESULTS

The implications of the Boivin-Vendrely idea of constancy of DNA to the appearance of stained cytological slides are obvious: the larger the nucleus, the more lightly colored it should appear. That this is approximately true is evident when any section of an organ is examined. But visual estimates can never be relied upon to show whether this dilution

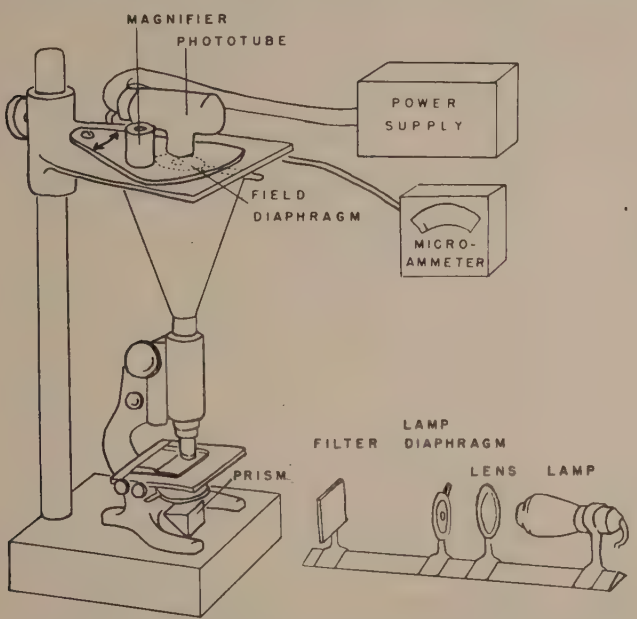

Fig. 1 Diagram of apparatus for measurement of the absorption of microscopic preparations. An optical bench carries a lamp, bullseye lens, iris diaphragm, and filter for isolating a narrow spectral region (monochromatic light). In place of the conventional mirror, a 90-degree prism reflects light into the optical path of the microscope. Some distance above the microscope is an iris diaphragm which is centered with respect to the microscopic field. Above this diaphragm is a plate which carries a focusing magnifier and a phototube, which may be interchanged in position above the diaphragm by rotation of the plate. In practice the object which is to be measured, while viewed with the magnifier, is centered in the iris, and the latter then closed to circumscribe the exact area for measurement. The phototube is then moved into position above the diaphragm, and the microammeter deflection noted. With the mechanical stage the slide is then moved so that an empty area is in the field of measurement, and a second reading is recorded. The transmission (T) is the first reading divided by the second.

of color may actually be accounted for by increase in nuclear volume; this must be done by computation from photometric measurements.

This relation of DNA to changing nuclear volume is well illustrated by spermatid nuclei, which shrink as differentiation progresses toward the mature sperm nucleus (Pollister and Swift, '50). The measured extinctions (fig. 2) are in

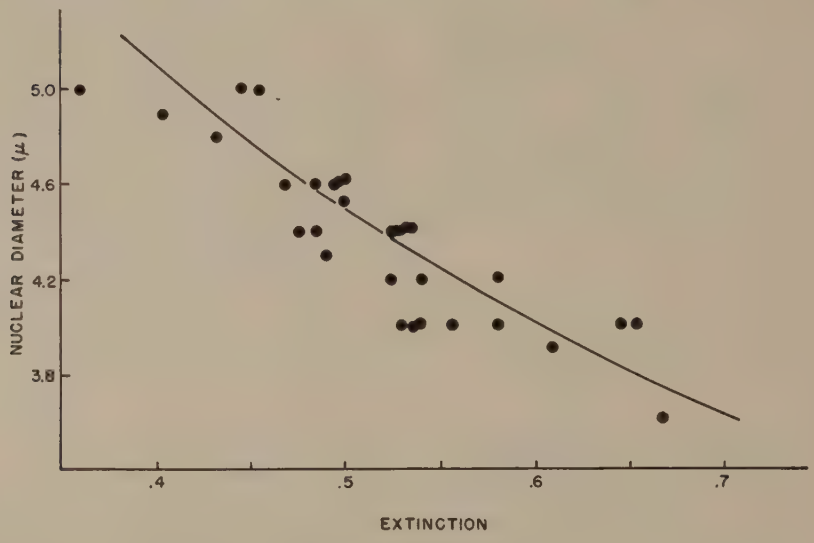

Fig. 2 The relation between nuclear size and extinction (optical density) of 32 Feulgen-stained spermatids of the rat. The solid line shows the theoretical relationship between nuclear diameter and extinction, if the number of absorbing molecules is constant (after Pollister and Swift, '50).

fairly good agreement with a curve which has been computed on the assumption that with decrease of nuclear size there is an exactly compensatory increase in concentration, that in other words, there is no actual change in amount of DNA (or Feulgen dye). The nuclei of adult differentiated tissues, if the Boivin-Vendrely view is correct, must represent the end results of changes of this sort leading to nuclei of various sizes, from the small dense ones of normoblasts to such large diffuse types as neurons, which are over 20 times as large.

The first project in attempting to test the view of DNA constancy by direct measurements of individual nuclei was to follow the DNA throughout all the significant stages in the life cycle of one animal, the mouse (Swift, '49, '50; Alfert, '51).

Measurements were made on large samples of interphase nuclei of mouse tissues, which had been fixed so that differences in chromatin distribution were minimal. When the arbitrary mean DNA (or Feulgen) amounts were computed it was found that there was one value which occurred in all the tissues, and which in most tissues was the only value (table 2). These

TABLE 2

DNA content of class I nuclei

CELL TYPE	NUMBER OF NUCLEI MEASURED	DNA FEULGEN $\left(\frac{Er^2}{F}\right)$
Liver	21	3.34 ± 0.05
Pancreas	20	3.10 ± 0.06
Thymus	33	3.28 ± 0.06
Lymphocytes	19	3.20 ± 0.08
Sertoli cells	18	3.00 ± 0.12
Kidney tubule	30	3.14 ± 0.04
Epithelium of small intestine	20	2.97 ± 0.04
Spleen	33	3.12 ± 0.04
Neurons (spinal cord)	20	3.14 ± 0.07
Interstitial cells of testis	20	3.05 ± 0.08

nuclei were called DNA class I. The differences among these class I nuclei are not great, although they are in some cases statistically significant. These significant differences may mean that there are slight variations in the range from tissue to tissue, about which more can be learned if the methods are made more accurate. On the other hand, it is felt that these differences are actually well within the over-all error of the present method of cytological photometry. The point to be emphasized is that these are very slight variations in comparison with the gross differences which are found in some other DNA classes, which accompany changes of chromosome number.

In several tissues, for example hepatic parenchyma, there are large nuclei, obviously much more heavily stained than they could be if the DNA of the smaller class I nuclei were diluted. These nuclei, called classes II and III, are found to contain two and 4 times as much DNA (or Feulgen) as the class I nuclei (fig. 3, solid line curves). These three DNA classes in adult liver are cleanly separable; and no nuclei are found which have amounts intermediate between classes.

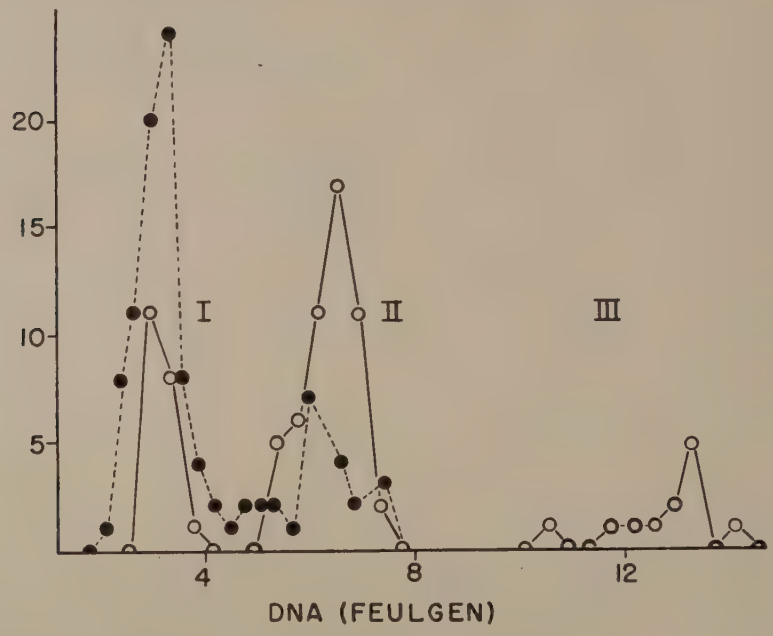

Fig. 3 Amounts of DNA, in arbitrary units, in individual mouse liver nuclei, as indicated by the Feulgen reaction. (——, adult; – – –, 10 days old.)

The relationship of these somatic DNA values to that of the gametes has been studied (table 3). In the testis there are two classes of cells which are not recognizable as any stage of meiosis, but precede the appearance of meiotic stages. One of these has the class I amount, the other class II. The earliest primary spermatocytes likewise are class II; and it is therefore assumed that these are developed from the class II

premeiotics. The two maturation divisions result in distributing the class II amount equally to the 4 spermatids, each therefore receiving one-half the class I amount (table 3). The total amount for the 4 spermatids is present before the meiotic process begins.

The above results are summarized in table 4. There are 4 distinct DNA classes among mouse nuclei, which are simple

TABLE 3

DNA content of stages in spermatogenesis

CELL TYPE		NUMBER MEASURED	DNA-FEULGEN
Premeiotic	class I	33	3.28 ± 0.07
	class II	78	5.96 ± 0.07
Primary spermatocyte		41	6.28 ± 0.07
Secondary spermatocyte		30	3.35 ± 0.04
Spermatid		28	1.68 ± 0.02

TABLE 4

DNA content and chromosome complement

DNA CLASS	NUMBER OF NUCLEAR TYPES	MEAN DNA-FEULGEN	PRESUMED CHROMOSOME COMPLEMENT
Spermatid	1	1.68	haploid (N)
I	12	3.16	diploid (2N)
II	6	6.30	tetraploid (4N)
III	2	12.8	octoploid (8N)

multiples of one another: the spermatid amount which therefore characterizes nuclei with the haploid chromosome number and presumably a single set of genes; class I nuclei found in all 12 tissues, with twice the spermatid amount, and therefore corresponding to the diploid chromosome number and double gene complement; classes II and III with respectively 4 and 8 times the sperm amount, which, since they are larger nuclei and are found in organs in which polyploidy has been repeatedly described, seem to correspond to 4 and 8 sets of chromosomes.

There is no overlap of DNA classes in tissue in which cell division is rare; but intermediates between classes are readily detected in embryonic organs where cell divisions are numerous. In such a tissue, nuclei which appear structurally no different from interphases of adult tissue nevertheless do not fall into one or two distinct classes. Instead, a range of values is found, covering the extremes of classes I and II and intermediate values as well (fig. 3, broken-line curve). All recognizable very early prophases which have been measured have been found to fall in the class II or tetraploid range of DNA content, and late telophases have the diploid class I amount. From this it appears that the synthesis of the new DNA must take place during interphase and be completed before the visible onset of prophase. The ensuing mitosis merely apportions equally between the two daughter nuclei the amount formed in interphase.

It has been found that in oögenesis, as in spermatogenesis, the synthesis of DNA for the polar bodies and egg nucleus takes place very early, before striking maturation phenomena (Alfert, '51). The oöcytes start from very small cells, and the nuclei eventually become enormous in the large oöcyte. These large nuclei have often been called Feulgen-negative, implying that they contain no DNA. Actually the very small oöcyte nuclei, like the premeiotic spermatocytes, already have the tetraploid amount of DNA, all that is needed to produce polar body nuclei and a haploid amount for the egg nucleus (table 5, compare with values of table 3). As the oöcyte nucleus swells this original DNA becomes greatly diluted, but the same total amount is to be found in nuclei 15 times as large, beyond which the DNA cannot be reliably estimated photometrically. There is no evidence of decrease of DNA.

The DNA content has been measured in 20 male and female pronuclei of fertilized mouse eggs. In this form there is no fusion to a single zygote nucleus, and side by side the two pronuclei produce chromosomes which go onto the first division spindle. All the measured pronuclei had the morphological aspect of interphases; but, as would be expected from

the fact these were about to undergo mitosis, the DNA amounts fell within a haploid through diploid range (fig. 4). The male and female pronuclei in any one embryo were found to have very nearly the same amount of DNA. From this fact, and from the tetraploid amount found in the oöcytes, it is clear that, as in the case of the sperm nucleus, the egg nucleus immediately after the reduction divisions must have the haploid amount of DNA.

The results of study of a series of early cleavage nuclei are shown in figure 4. As just stated, the amounts in the two pronuclei are sufficient to bring the DNA of the fertilized egg

TABLE 5

DNA content of mouse oöcyte nuclei

DESCRIPTION	NUMBER MEASURED	MEAN VOLUME	MEAN AMOUNT
a. Early peripheral oöcytes with a follicle of a single layer of flattened cells (10-day-old mouse)	15	438 μ^3	6.45 ± 0.14
b. Medium-sized oöcytes with a follicle of a single layer of cuboidal cells (10-day-old mouse)	15	1365 μ^3	6.46 ± 0.16
c. Large oöcytes with a follicle of 2–3 layers of cells (10-day-old mouse)	20	6010 μ^3	6.68 ± 0.18

up to the class II, or tetraploid value. The result is that, at first cleavage, each of the nuclei receives the class I or diploid amount. DNA values in these rapidly cleaving embryos range from the diploid class I value to double this amount, the latter of course being preparation for a division (fig. 4). There is no evidence that, as has been suggested from visual inspection in other forms, the DNA content of early cleavage nuclei of the mouse is low and rises in later cleavages. Of course the smaller nuclei of later cleavages *look* darker, for the DNA is concentrated in a much smaller volume. But when objective photometric measurements are made, and this volume change taken into account in the computations, it is evident

that there is no change in amount of DNA per nucleus, other than that incident to nuclear division. One can now, of course, complete this cycle of DNA in the mouse by jumping all the way to adult tissues, where, as already described, this class I amount characteristic of the cleavages has been found in all the most common diploid tissue nuclei. (The polyploid classes II and III are a result of a fairly late differentiation, mostly beyond the embryonic stage.)

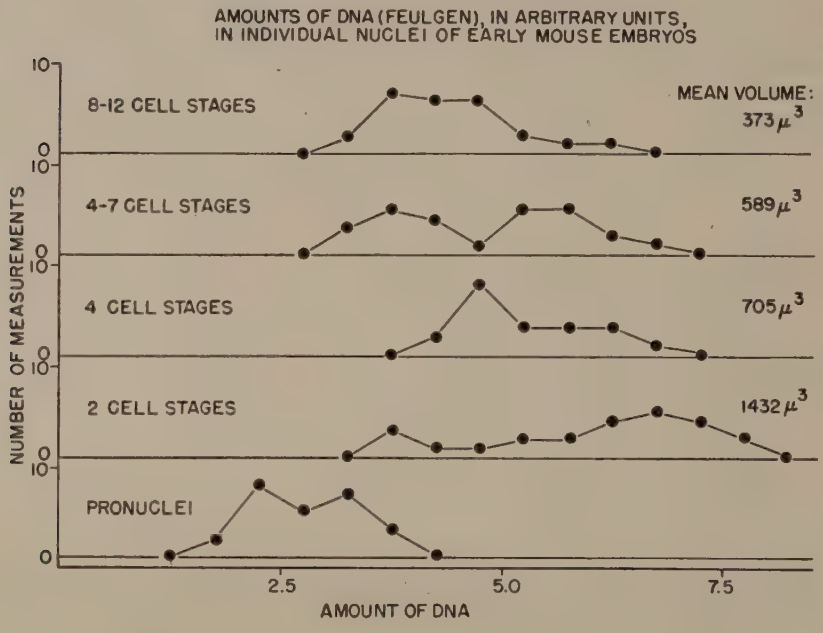

Fig. 4 Amounts of DNA (or Feulgen), in arbitrary units (Er^2/F), in individual nuclei of early mouse embryos (after Alfert, '51).

The major results on the mouse have recently been confirmed on tissue of three other animals, the frog, bull, and grasshopper (Swift, '50).[1] It is interesting that in the malpighian tubules of the grasshopper there is evidence of a

[1] This part of the work was carried out at the University of Chicago, aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

class IV nucleus, in which the DNA content is in a range of 16 times the haploid amount. Results essentially similar to these have been reported from other laboratories. In the rat, Ris and Mirsky ('49) and Pasteels and Lison ('50) have reported the 1:2:4 ratio of DNA amounts in the polyploid series of liver nuclei. The latter authors found the DNA content of a number of rat diploid nuclear types to be, as in the mouse, nearly exactly twice that of the haploid nucleus of the spermatid.

DISCUSSION

There are two reports of departures from the strict correlation of DNA content with chromosome complement. The values of Pasteels and Lison for diploid nuclei of adipose tissue, hepatic parenchyma, and pancreatic epithelium were as much as 30% less than twice the spermatid amount. Schrader and Leuchtenberger ('49) have reported some values for plant nuclei which are difficult to reconcile with the data on animal tissues.

With these two exceptions the results of measurement of the DNA content of individual nuclei by the Feulgen reaction are in agreement with expectation from the Boivin-Vendrely hypothesis. The amount of desoxyribonucleic acid per nucleus appears to be parallel with the number of sets of chromosomes, and, presumably, also with the number of sets of genes.

If it is the rule that the amount of DNA per nucleus falls within a constant range which is closely related to the chromosome complement, then determinaton of DNA can be a valuable tool in cytology and biochemistry. Many cytological problems involving degree of polyploidy or polyteny, time of chromosome duplication, and other factors can profitably be re-examined. As Davidson and Leslie have pointed out, this principle of constancy of DNA can also be of great value in biochemical analysis, for one need only divide the total DNA by the amount per nucleus (determined independently by the Boivin-Vendrely technique) in order to arrive at an estimate of the number of cells in the analytical sample. By

reference to this as a base, the analytical values for other substances can likewise be expressed as amount per cell.

The data presented are consistent with the conclusion that DNA may be one major component of the gene. There are several other lines of evidence which are best interpreted as support for this view. *First,* wide qualitative application of the Feulgen reaction has shown that DNA, like the genes, is present in all sorts of chromosomes, and that it does not occur outside the chromosomes, or their equivalent in lower organisms. *Second,* tracer studies clearly indicate that DNA is unusually stable, being little if at all changed during ordinary day-to-day cellular metabolism, and that new DNA is formed in quantity only when there is chromosome reproduction (Brues, Tracy, and Cohn, '44; Brown, '48). *Third,* the phenomenon of directed mutation is a specific function of DNA which is remarkably like that expected to be characteristic of genic substance. *Fourth,* the action spectrum of mutation production by ultraviolet radiation resembles the characteristic absorption spectrum of desoxyribonucleic acid.

It thus appears that many of the commonly accepted properties of gene may be explained on the assumption that DNA is an essential genic component — that we are perhaps, therefore, close to the long-sought goal of grappling with the gene chemically as well as biologically. In a sense, this is very gratifying; but, on the other hand, it must be conceded that it is disappointing that this particular line of chemical investigation appears to have completed a circle and brought us back to the point where we were some time ago, the only difference being that perhaps we can substitute DNA for chromosome or gene. But, as Schultz ('41) said, "We must think of the gene as having two faces, one turned toward the maintenance of its own integrity, one looking toward the synthetic metabolism of the cell." It is the former with which most of what has just been summarized is concerned. A "DNA-gene" is constant and it apparently does self-duplicate, but this very constancy leaves us still on the horns of the old differentiation dilemma. If the "DNA-gene" con-

tents of a mammalian hemoblast and a neuroblast are identical how are we to explain the fact that in one case this complex leads to a tiny, non-nucleated sac of protoplasm, in the other to a huge cell with a cytoplasmic mass hundreds of times the volume of its nucleus? Here we are just about as much dependent on speculation as we always were. Is the DNA macromolecule not only a self-duplicating gene with specific properties, but also a sort of universal factory which will make different products in varying amounts according to some extraneous influence, such as supply of substrate? Or, is the DNA actually only a sort of constant framework of the gene, while in the specific synthesis of phenotypic expression we are seeing evidence of the activity of some other, more metabolic, part of the gene, as yet unidentified? It has been shown in clearest fashion that experimentally the two gene functions are separable. The specific syntheses go on in nitrogen-mustard-treated tissues long after DNA reproduction has stopped (Bodenstein, '47, '48). Furthermore, an interesting case of natural occurrence of greatly increased synthesis without any accompanying change in DNA content has recently been described by Schrader and Leuchtenberger ('50). Perhaps evidence of this sort favors the second possibility, that important genic components other than DNA remain to be discovered.

OPEN DISCUSSION

Chairman Mazia: The paper of Dr. Pollister is before you for discussion.

Dr. Carlson: I should like to ask Dr. Pollister a question. With regard to these two classes of premeiotic cells you showed on the screen, I assume that one class is for the early interphase and the other class for the larger amount of the late interphase.

Dr. Pollister: They are both interphases. The first one is predominant in a very early mouse testis long before there is any differentiation. In the testes of older animals cells with larger amounts appear, and then the earlier diploid ones

decrease progressively in number. Many interphases with the tetraploid amounts appear in matured testis, and we assume that those go over to the primary spermatocyte.

Dr. Carlson: You do not interpret this increase in the DNA as synthesis during this interphase.

Dr. Pollister: We do not know how these are related to one another. It may be an end of mitosis.

Dr. Carlson: I was leading up to a question with regard to the meiotic prophase. Have you made any measurements during meiotic prophase to see of there is any synthesis of DNA during this long growth period that you term a prophase; since you did make the statement that presumably in mitotic prophase there is no synthesis of nucleic acid, DNA, during prophase, but probably during the preceding interphase.

Dr. Pollister: This method breaks down when you try to measure a nucleus in which the chromatin is concentrated in a few lumps with large empty spaces between them, as is the case during the later stages of a prophase. You cannot evaluate the error introduced by these differentiations but you can tell what direction that error must take. We are assuming that there is no change, from the fact that in the earliest primary spermatocytes as well as the earliest meiotic prophase, you find this tetraploid amount.

The secondary spermatocytes at the interphase, which is the time we try to measure them, have half the amount of DNA found in early primary spermatocytes. There could, of course, be a rise and then a fall, somewhere between early first prophase and interphase before the second division. We have no way of detecting such a change. Microchemistry might detect it, but it is not possible to answer this by microspectrophotometric studies.

Dr. McCrady: I would like to ask another question about technique. When you make the photometric measurements through a cylinder of the nucleus, you assume that you are going the whole way through, and your sections will have to

be pretty close to the diameter of the nucleus in order to avoid the possibility of having one part above another.

Dr. Pollister: Yes, they are always one or two microns thicker than the nuclear diameter.

Dr. McCrady: When it is in that neighborhood, there is the obvious likelihood that in some cases you have a little slice dropped off the nucleus, so that you would be measuring less than the full diameter.

Dr. Pollister: There is no likelihood at all of it. These were examined with that in mind.

Dr. McCrady: Do you examine in sections, or how?

Dr. Pollister: You focus on the top and the bottom of the nucleus, and you soon become able to distinguish the upper or lower nuclear surface from a sliced-off top or bottom. In the beginning one will occasionally measure such an incomplete nucleus, but the higher transmission obtained in such a case is a good indication that something is wrong, and a recheck detects the cut-off nucleus.

Dr. McCrady: I should think it would be easier to see the lid. The question in my mind was, could you see the absence of the lid?

Dr. Pollister: You can go right up and see the upper surface. It is a thing a cytologist seldom has to do unless he is making photometric measurements, but it is quite easy.

Dr. Moses: Getting back to the question of whether DNA is synthesized, we have been doing DNA determinations on *Trillium* anthers. The situation there is a rather fortunate one, since meiotic divisions are more or less synchronous. Even within the anthers of a single plant, you can get quite a lot of material which is very nearly in the same stage of division.

Dr. Steele at Brookhaven has worked out a rather nice semimicro modification of the Snyder method. He has done these analyses and so have we. We have found that in the pachytene stages, as compared with the later diakinesis and metaphase stages, there is an apparent increase. There is a difference of about 30% bewteen the amount of DNA present

per nucleus in the pachytene stages and there is that much more in the metaphase.

We also have data which indicate that, as soon as the two divisions are complete and the tetrad stage has been reached, there appears to be again an increase in the amount of DNA in terms of pollen mother cells.

This agrees with what Dr. Sparrow found when he extracted *Trillium* pollen mother cells with molar sodium chloride and measured the extinction of the extract at 2600 A units. He found there was no change throughout the two divisions.

Dr. Rietz: I want to ask Dr. Pollister what fixation was used on this material.

Dr. Pollister: Most of this material was fixed in strong formaldehyde, which cuts down the distributional error considerably. We could not have done this so well with acetic-alcohol fixation.

I have some unpublished data here given me by Dr. Swift about the frequency of polyploidy in beef tissues, and beef liver, particularly. That is the big discrepancy between the values which Mirsky and Ris reported and those of Boivin and Vendrelys; the liver value obtained by Mirsky seemed to be nearly three times that of the bull sperm.

The values for other tissues can be brought into line very well if you accept the sperm value of Boivin and the Vendrelys as being more nearly correct than the Mirsky values. Then the actual determinations of DNA per nucleus are pretty close except for those of beef liver.

Swift has counted the polyploid nuclei in beef liver in sections. About 30% of the liver nuclei, of course, are not the parenchyma nuclei, but are the nuclei of the walls (blood vessels). There are two different kinds of parenchyma nuclei. In a 2-year-old bull the number of polyploids is 3% of the total nuclei, and this can raise the value of the DNA per nucleus in a population of some billions of nuclei very little. Polyploidy cannot account for any great discrepancy. It could possibly account for a considerable discrepancy when you come to mouse nuclei, in which, in an old mouse, con-

siderably more than half the nuclei may be tetraploid. We are a bit puzzled by the fact that Boivin and the Vendrelys do not seem to have picked this up in mouse livers, although they have analyzed more mouse livers than any other organ. They have obtained a distinctly higher value for mouse liver, but it is not as high as we should expect. Possibly they were using young animals. The ploidy increases progressively throughout life. It is supposed to arise by fusion of two nuclei after a division.

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PRECURSORS OF NUCLEIC ACIDS[1]

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NINE FIGURES

The importance of nucleoproteins to processes of growth and development has been assumed and we have set out to learn something of their precursors with two main goals in mind; first, to find out what compounds are involved in the biosynthesis of nucleoproteins, and second, to investigate structural analogs of those compounds in a search for antimetabolites which could interfere preferentially with rapidly growing tissues. Of necessity most of our attention has so far been focused on the first point.

The knowledge, furnished by Plentl and Schoenheimer ('44), that guanine, uracil, and thymine were not utilized by the rat for nucleic acid production was the starting point for these investigations. The purine, adenine, was investigated because of its ubiquitous occurrence not only in both types of nucleic acids but also in coenzymes and in adenosinetriphosphate (ATP). The convenient synthesis of Baddiley, Lythgoe, and Todd ('43) was first used to incorporate N^{15} into the 1- and 3-positions of adenine (Brown, Roll, Plentl, and Cavalieri, '48).

This adenine was administered to rats and it was not only incorporated as adenine but its isotope was also incorporated into polynucleotide guanine (fig. 1). Degradation of the isolated guanine showed that the isotope was still in the 1- and

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3-positions of the purine ring. It was thus implied that the purine skeleton was retained intact during this transformation of adenine into guanine.

Recently Kalckar has reported ('49) that adenine, containing C^{14} in the 8-position, is also converted into guanine by *Lactobacillus casei.* With the same adenine-8-C^{14}, we have recently obtained the same kind of result in the rat and in yeast (Marrian, in press; Kerr, Seraidarian, and Brown, '51). These samples of guanine have not yet been degraded to show the position of the isotope, but the fact that this "far corner" of the ring goes along too furnishes further circumstantial evidence that the purine ring system remains intact during the transformation.

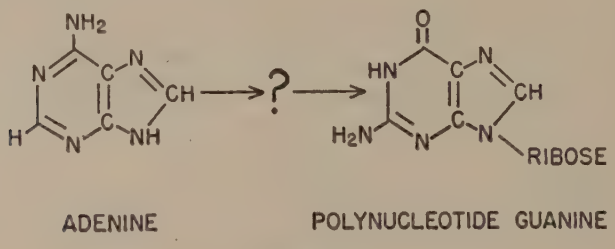

Figure 1

The striking difference between the results with adenine and the original observations with guanine prompted repetition of the administration of guanine with our Sherman strain rats (Rockland Farms), and completely confirmed the negative findings of Plentl and Schoenheimer ('44). Guanine was also tested by intraperitoneal administration and the negative results of the oral administration were duplicated. Reinvestigation of guanine was started again last year when Kidder, Parks, Dewey, and Woodside ('49) reported the tumor inhibitory action of azaguanine, the triazole analogue of guanine, and made predictions regarding a specific utilization of guanine by tumor tissue.

The previous year Cavalieri had prepared a number of the triazole analogues of purines, including azaguanine, and

they had been tested by Stock, Suguira, and others in the Chemotherapy Division of this Institute. Some toxicity but no tumor specificity had been observed. Azaguanine, the one in question, had been negative as far as specific effects were concerned on 4 tumors. After the report from Kidder's laboratory these assays were repeated and the series of tumors was extended. The current spate of reports (Stock et al., '50; Sugiura et al., '50; Gelhorn et al., '50; Law, '50; and Sokoloff et al., '50) on the effects of azaguanine on tumors indicate that the inhibitory action of azaguanine is not universal.

A tumor on which there was agreement on the inhibitory effect was the transplantable mammary adenocarcinoma

TABLE 1

Incorporation of labeled guanine in nucleic acids in normal and tumor-bearing mice [1]

Atom per cent excess N^{15}

	NORMAL	BEARING Eo 771		
	Viscera	Viscera	Tumor	
			PNA	DNA
Purines		0.049	0.058	0.046
Guanine	0.090	0.072	0.105	0.050
Adenine	0.026	....	0.037	0.038
Pyrimidines	0.027	0.024		

[1] C57 black mice receiving guanine, I.P., 0.8 m*M*/kg, 5 of 6 days.

Eo771 in mice of the C57 black strain. This is a solid tumor from which it is possible to obtain reasonably large and representative samples of tumor tissues. We have studied the incorporation of intraperitoneally administered guanine in mice bearing this tumor. The results (Brown, Bendich, Roll, and Sugiura, '49) indicate a small but definite specific incorporation of guanine into the nucleic acids of the normal tissue of that mouse, represented by the combined viscera, and into both the pentose nucleic acid (PNA) and desoxyribonucleic acid (DNA) of the tumor. The analogous experiment in the same strain of mice without tumor showed almost identical guanine utilization so the result cannot be attributed to the presence of the tumor (table 1). Oral administration

of guanine has given a similar picture, with slightly lower values.

The small amount of N^{15} found in the adenine might raise the question of whether there is any conversion of the guanine into adenine. The guanine used contained N^{15} in the substituent 2-amino group as well as in the 1- and 3-nitrogens of the ring. The portion which went to allantoin contributed this 2-amino group to body pool ammonia so that there is always an appreciable amount of isotopic nitrogen in all the nitrogenous compounds. Drawing conclusions from 9 experiments with guanine in mice and rats, 7 of ours and 2 from Plentl and Schoenheimer, we can say that this "base-line" is about one-half as large when the guanine is administered orally, which means slowly throughout the day, as when it is administered intraperitoneally in one single daily dose. It is probable that the N^{15} in the adenine, and, of course, part of that in the guanine, arrived by this route, and there is no particular reason to suspect that guanine is being converted into adenine. Experiments with C^{14}-labeled guanine should answer that question explicitly.

Recent feeding of adenine to the C57 mice shows their utilization of adenine to be about one-fifth that of the Sherman rat, thus indicating a decided quantitative difference. With the present figures it would appear that there is a qualitative difference in guanine utilization by the two species, but it is conceivable that this is only a large quantitative difference, and only by the use of the more sensitive C^{14} as a tracer will it be possible to find out whether there may not be a trace utilization of guanine in the Sherman white rat. It can be hoped that purine utilization in mammalian species in general will not prove to vary as widely as does the utilization of purines by bacteria!

In most of our experiments, especially those where a large number of purines have been surveyed, we have used the total viscera in order to be able to obtain more isolated nucleic acids from a given amount of dietary compound. In dealing with heavy isotopes, and most of our work has been

with N^{15}, the mass spectrometer prefers about a milligram of nitrogen, which means that at least 2 to 5 mg of an isolated compound is necessary for adequate characterization as well as isotope analyses. Essentially complete extraction of the mixed nucleic acids from tissues has been attempted and from this nucleic acid preparation individual compounds for analysis have been isolated.

Study has now been made of the incorporation of adenine into the total nucleic acids of various organs of the rat. Relative renewal in different organs varies markedly (Furst,

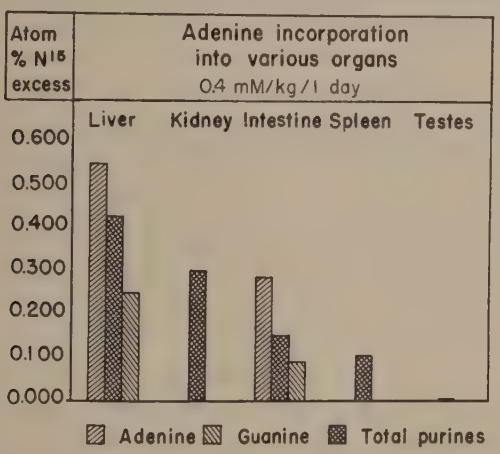

Figure 2

Roll, and Brown, '50) (fig. 2). The polynucleotide adenine generally contains about twice as much N^{15} as the polynucleotide guanine, so that the apparent guanine renewal, with adenine as a precursor, is about half the apparent renewal of adenine.

The presence of several different PNA's in the cell has been assumed and, since there are as yet no chemical methods available for fractionating the individual entities, all the PNA in one fraction has been sought. The work of Hammarsten's group with glycine (Bergstrand et al., '48), their comparison of the PNA associating isolated nuclei with the

rest of the cytoplasmic PNA, shows that, from the standpoint of relative renewal, there are at least two types of PNA in liver tissue.

An adaption of the Schmidt and Thannhauser method ('45), which had been critically evaluated by Schneider ('46), was used for the separation of PNA and DNA. PNA and DNA were first separated from the combined viscera and it was found that there was a strongly preferential incorporation of isotopically labeled adenine into the pentose nucleic acids as compared with the desoxypentose nucleic acids (Furst, Petermann, and Brown, '48). The isotope content of the purines of the DNA fraction was only about 3.5% of that obtained in the PNA fraction, and the uncertainty involved in the colorimetric determinations of small amounts of PNA in the presence of large amounts of DNA made it impossible for us to know whether this isotope was due to actual renewal, or to contamination by PNA. In order to narrow these limits of uncertainty, the procedure used for the separation of the two types of nucleic acids was re-examined (Furst, Roll, and Brown, '50). The Schmidt-Thannhauser separation depends upon the lability of the PNA fraction in 1 *N* sodium hydroxide at 37°C. and the subsequent precipitation of the DNA fraction in the presence of trichloroacetic acid. The effectiveness of the procedure employed was tested by adding a sample of isotopically labeled yeast nucleic acid, prepared from yeast grown on isotopic ammonia, to a sample of normal rat liver. The DNA fraction which was first obtained contained 0.092% N^{15} (table 2). With the expectation of obtaining further purification, the precipitated DNA was redissolved in alkali and was again precipitated by acid. An orcinol test showed a small amount of PNA present in the supernatant from this reprecipitation. The reprecipitation of DNA, now containing only 0.021% N^{15}, had thus reduced the contamination by a factor of 4. Isotope concentration in the purines from the final DNA fraction (0.027% N^{15}) was 1.3% of that in the purines of the PNA fraction (2.11% N^{15}). It cannot necessarily be interpreted that all this isotope found in the

DNA fraction is due to contaminating PNA, since yeast nucleic acid samples always contain a trace of material responding to the diphenylamine test for DNA, and it has been demonstrated that a highly polymerized DNA may be obtained from yeast. A sample of yeast nucleic acid used here, while essentially PNA, gave a response indicating $0.46 \pm 0.4\%$ DNA in the diphenylamine test. If it were assumed that this DNA was labeled, and of a sufficiently high molecular weight to appear in the DNA fraction, one would expect to find considerably more N^{15} in the DNA fraction than was actually found. Whether the estimate of the amount of macromolecular DNA in the YNA sample or the estimate of the extent to

TABLE 2

Isotope analyses: fractionation of liver nucleic acids plus labelled YNA

	ATOM PER CENT EXCESS N^{15}	CALCULATED ON BASIS OF 100% IN PURINES OF PNA
Copper purines from PNA fraction	2.11	100.
DNA; first precipitation	0.092	(4.4)
DNA; second precipitation	0.021	(1.0)
Copper purines from twice pptd. DNA	0.027	1.3

which that DNA is labeled is at fault, it is evident that it is impossible to estimate correctly the relative contributions of the yeast DNA and contaminating PNA to the isotope content of the final DNA preparation. However, since there was such a definite removal of PNA between the first and second precipitations, some portion of the apparent contamination is probably due to PNA still remaining in the reprecipitated DNA fraction, and we can say that the adequacy of the separation seems good to within about $1 \pm 0.5\%$.

Studies of the renewal of the nucleic acid purines in only the livers of fully adult (ca. 350-gm) rats were then carried out. In such rats liver tissue is essentially nongrowing, so that very few new cells or new nuclei are being formed, and the relative incorporation into the purines of the pentose and desoxypentose nucleic acids was in the ratio of 73 to 1 (fig. 3). The

observed metabolic result could be due either to the fact that adenine does not serve as a precursor of the DNA purines as suggested by Reichard ('49), or it could be due to the fact that the DNA purines are simply not in a rapid dynamic equilibrium.

An experiment in which adenine was fed to partially hepatectomized rats furnished satisfactory proof that adenine may serve as a precursor of desoxypentose nucleic acid purines. In the rapidly regenerating livers it is certain that extensive mitosis and production of new nuclei, and therefore of new DNA, is occurring. During the 5-day period of rapid growth, the incorporation into the DNA purines of the liver was about 75% of that into the PNA purines.

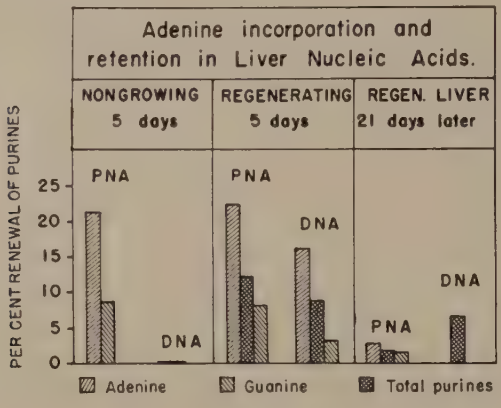

Figure 3

In yet a third experiment in this series, partially hepatectomized rats were fed isotopic adenine for 5 days to bring the isotope contents to approximately the values in the second experiment. They were then allowed normal food, with no isotopically labeled supplement, for an additional three weeks. At the end of that time the DNA purines still retained a very high isotope content, while the continuing metabolism of the PNA purines had resulted in extensive replacement by nonisotopic purines elaborated *in vivo*. This

again confirms the observation that there is a very slow renewal of the purines in nongrowing tissue.

These relative renewals, when adenine labeled in the ring only is serving as a precursor, parallel but considerably exceed the differences shown in any of those studies in which isotopic phosphate (Hevesy, '48) or N^{15} glycine (Bergstrand et al., '48) had been studied as precursors. The divergence between results with phosphate and adenine might be attributable to the experimental difficulties (see von Euler et al., '48; Davidson et al., '49; and Kelly and Jones, '50) due to non-nucleic acid phosphorus, but those between glycine and adenine remain obscure. In the glycine experiments of Hammarsten and co-workers significant incorporation of glycine nitrogen into the purines of the DNA was observed even in the nongrowing liver. The two methods in use for the separation of the PNA and DNA fractions are somewhat different, but neither seems open to criticism per se. Pending completion of experimental work with both precursors under identical experimental conditions, it is rather difficult to produce any rational explanation of these differences between glycine and adenine as precursors of DNA.

From our relative renewals with adenine it is conceivable that all the adenine incorporated into the DNA purines was introduced in the process of formation of new nuclei and that, once formed, the carbon-nitrogen skeleton of the DNA is not in dynamic equilibrium with other constituents of the cell. Observations on desoxyribonuclease inhibitors, in the laboratories of Chargaff ('48) and Laskowski ('49), are perhaps pertinent to this slow renewal of DNA in the cell.

The implications of the fact that the desoxypentose nucleic acids seem to be an exception to the general concept of a dynamic equilibrium of tissue constituents are not obvious. However, Schrödinger ('48) has pointed out from the purely objective viewpoint of a theoretical physicist that it would be desirable for the genetic material to possess a stability of an order which the physicist cannot correlate with even random heat motion of molecules, much less with the chance

variations which could occur during the continuing metabolism of substances which are in dynamic equilibrium.

Most of our work has been based on the isolation of the individual purines from the PNA fraction. However, the recent recognition by Carter and Cohn ('49) of the existence of two adenylic acids in hydrolysates of pentose nucleic acids has raised the question of whether these two adenylic acids are simply products of the chemical hydrolysis of pentose nucleic acids or whether they can be distinguished metabolically. In current experiments with adenine-8-C^{14}, prepared according to Clark and Kalckar ('50), D. H. Marrian is isolating 6 ribonucleotides, including the two adenylic acids and two guanylic acids, by ion-exchange chromatography (Cohn, '49, '50). We have adapted this ion-exchange chromatography technique to the separation of the total pentose mononucleotides as obtained from rat tissue, in what is essentially a Schmidt-Thannhauser supernatant. The small differences now being observed between the two adenylic acids and the two guanylic acids are of doubtful significance and indicate that these different nucleotides of adenine and of guanine are metabolically indistinguishable. The adenylic acids contain about 4 times as much isotope as the guanylic acids and the pyrimidine nucleotides are without activity (fig. 4). The ratio between the renewal of the adenylic and guanylic acids is somewhat different from that reported for their phosphate by Carter and Volkin ('49).

We have also surveyed all the purines normally associated with mammalian metabolism plus several less usual ones (Brown, '48). With all the oxypurines, hypoxanthine, xanthine, or uric acid, the only observed fate in the rat was extensive conversion to urinary allantoin. Incidentally, the proportion of the allantoin derived, which is probably a rough measure of the ease of oxidation to allantoin, increases in the order: hypoxanthine, xanthine, uric acid.

In the case of hypoxanthine there is considerable evidence from several sources which lends favor to the consideration of hypoxanthine serving as a precursor of adenine. However,

the orally administered hypoxanthine, once at a level of 0.2 m*M* per kilogram and once at the very high level of 1.5 m*M* per kilogram, gave no evidence of incorporation of the hypoxanthine into the rat tissue nucleic acids (Getler, Roll, Tinker, and Brown, '49).

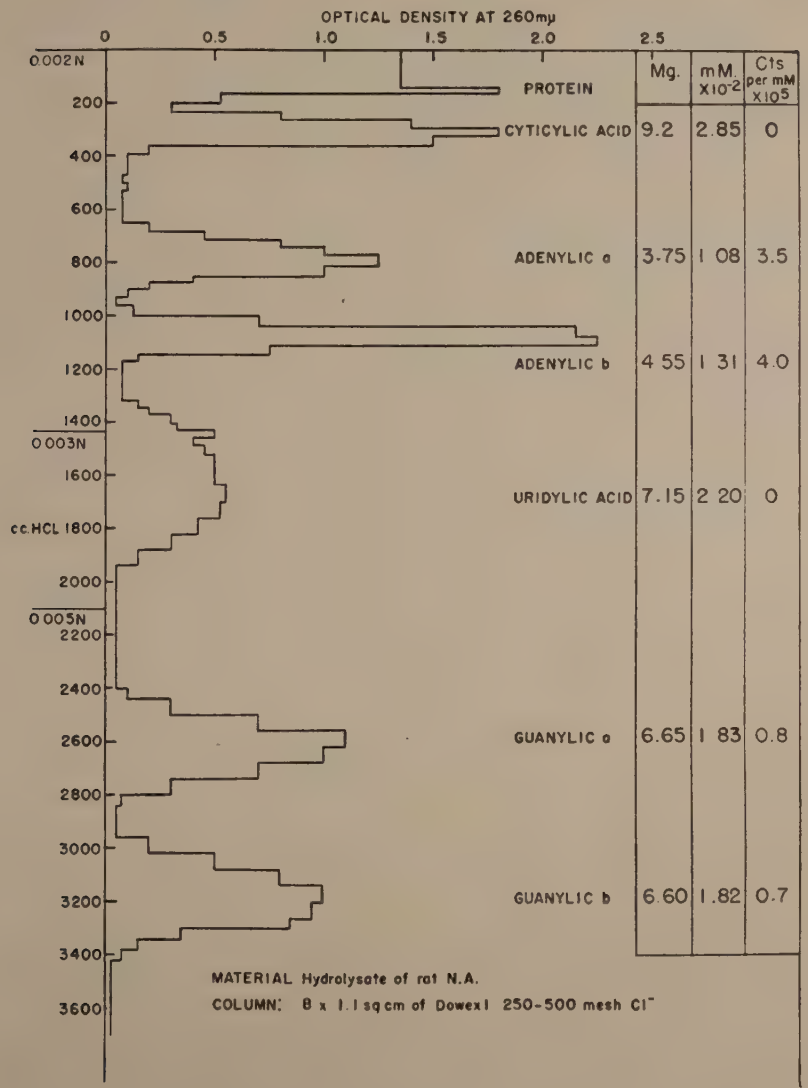

Fig. 4 Incorporation of adenine-8-C^{14} into individual nucleotides.

At present there is no satisfactory correlation between the known enzymes and the behavior of purines in the intact animal. The one characterized enzyme which is known to handle the riboside linkage in nucleosides is the ribonucleophosphorylase which has been so admirably described by Kalckar ('47). In *in vitro* studies it has been demonstrated that this equilibrium is far toward the synthetic side. The enzyme is effective only with hypoxanthine or guanine or their nucleosides, the purines which are not utilized by the rat (although guanine can be utilized by the mouse). The enzyme does not affect adenine, the purine which is extensively incorporated into ribosidic linkages by the intact animal.[2] Wajzer ('47, '49) has reported a concomitant secondary reaction which results in some synthesis of nucleotides from either adenosine or inosine, and he also reports that nucleotides can arise from a ribose-3-phosphate preparation and adenine, guanine, or hypoxanthine, but not from xanthine.

In an attempt to discover something about the mechanism of conversion of adenine into nucleic acid guanine, the two purines, 2-oxyadenine (isoguanine) and 2,6-diaminopurine, have been studied (fig. 5). Isoguanine was not incorporated into the tissue nucleic acids of the rat.

The other postulated intermediate, 2,6-diaminopurine, has not been reported from natural sources but it was utilized by the rat for the synthesis of guanine (Bendich and Brown, '48). In separate syntheses, isotopes of nitrogen and carbon have been incorporated into diaminopurine. The isotopic nitrogen was not only in the 1- and 3-nitrogens of the pyridmidine ring but also in the substituent 2-amino group. Each sample was fed to rats (Bendich, Furst, and Brown, '50), and in the first case the results with the N^{15} sample showed that the amount of polynucleotide guanine derived from 2,6-diaminopurine was comparable to that derived from dietary adenine. Diaminopurine was also catabolized to urinary allantoin and the labeled 2-amino group was contributed to body

[2] Dr. H. Kalckar ('50) has recently discussed the relationship between the results of enzyme studies and those in the whole animal.

pool ammonia, so that there was a small amount of N^{15} in most compounds. It was thus impossible to decide whether or not a small amount of direct conversion of diaminopurine into adenine had taken place since the isotopic nitrogen could arrive in the adenine by the second and indirect route. The sample with the C^{13} label permitted a much more conclusive demonstration that diaminopurine was acting solely as a precursor of guanine. An experiment with partially hepatectomized animals demonstrated that diaminopurine can lead to the guanine of either the pentose or the desoxypentose nucleic acids.

It must be emphasized that this transformation cannot proceed through free guanine since guanine itself is not utilized by these rats. Transformation of diaminopurine into poly-

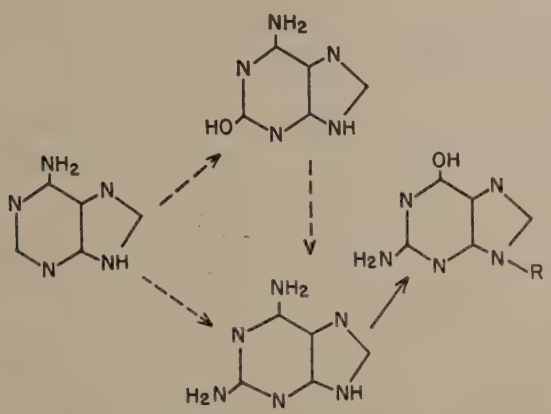

Fig. 5 Adenine, isoguanine, 2,6-diaminopurine and guanine.

nucleotide guanine involves two major alterations: the attachment of the pentose in the 9-position and the replacement of the 6-amino group by oxygen (fig. 6).

We cannot be certain whether or not 2,6-diaminopurine is a normal intermediate in the conversion of adenine into guanine. Reichard's ('49) demonstration that in certain instances the ring nitrogens of guanine may derive more N^{15} from glycine than do these of adenine makes it obvious that

a second anabolic pathway may give rise to guanine. It is not impossible that diaminopurine lies on such a pathway.

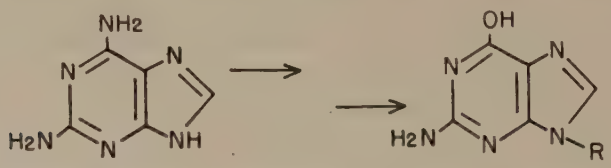

Fig. 6 Changes necessary in derivation of a guanine derivative from 2,6-diaminopurine.

It might be recalled that the renewal of guanine in the two guanylic acids is only one-fifth that of the renewal of adenine in the two adenylic acids, although as far as the phosphorus of these compounds is concerned the guanylic acids were renewed to about three-fourths the extent of adenylic acid phosphorus (Carter and Volkin, '49). These data could be used as further support for the idea that all the guanine is not derived from adenine if it be assumed that only complete nucleotides enter a nucleic acid. Also our data with the C57 mouse indicates that, in some cases, guanine itself may be utilized, and this might be an indication of yet a further pathway by which polynucleotide guanine may arise.

It is of interest that adenine and 2,6-diaminopurine are the most toxic of the purines and that they lead to two pharmacological effects which are profoundly different. The first demonstration of an inhibitory effect of 2,6-diaminopurine was that of Hitchings and co-workers ('48, '50) who showed that it could reversibly inhibit the utilization of adenine by *L. casei*. Several laboratories (Burchenal et al., '49; Hertz and Tullner, '49; Thompson et al., '49) have reported various significant pharmacological and growth-inhibitory effects which 2,6-diaminopurine can exhibit. Its administration to rats, dogs, man (Philips and Thiersch, '49), and swine (Cartwright and Wintrobe, '50) leads to profound changes, including severe denudation of the intestinal mucosa and an acute depletion of the hematopoietic elements in bone marrow. While certain of the toxic or inhibitory effects of di-

aminopurine can be reversed by the administration of adenine, it has been impractical to attempt to reverse the effect in mammals, because of complicating toxicity of large amounts of adenine.

The toxicity to mammals of large doses of adenine has long been known. The deposition of extensive crystalline masses in the kidney, after the administration of large doses of adenine, was observed in 1898 and these eventually proved to be 2,8-dioxyadenine (Nicolaier, '02).

We have studied the administration of large doses of adenine and observed that the crystals of 2,8-dioxyadenine are to be found in the tubules, initially in the distal tubules and in

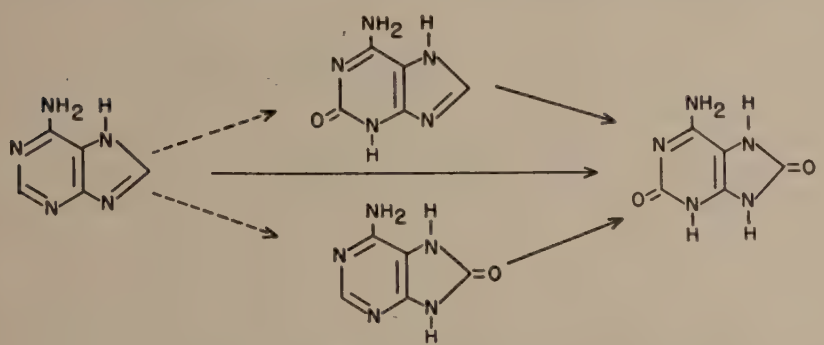

Fig. 7 Adenine, 2-oxyadenine, 8-oxyadenine, and 2,8-dioxyadenine.

extreme cases in both Henle's loop and proximal tubules. Isotopic adenine has been administered to rats (Bendich, Brown, Philips, and Thiersch, '50) in large amounts, 500 mg per kilogram, and the 2,8-dioxyadenine found in the kidneys was derived from the dietary adenine with but a very slight dilution of the isotopic nitrogen.

The possible intermediates (fig. 7) in the conversion of adenine into this 2,8-dioxyadenine were studied. The administration of labeled 2-oxyadenine, or isoguanine, in large quantity leads to the deposition of the kidney crystals, also with little dilution of the isotope. However, 8-oxyadenine was also tried and it, too, leads to 2,8-dioxyadenine so that we

cannot now make a choice as to whether oxidation may occur first in the 2- or 8-position in the normal course of metabolism.

Philips and Thiersch ('50) have made detailed pharmacological studies of adenine intoxication as well as parallel studies of isoguanine intoxication. The deposition of the extremely insoluble 2,8-dioxyadenine causes mechanical renal damage which, if sufficiently severe, may cause uremia and secondary changes in other organs, principally a depletion of the nucleated erythroid elements in the bone marrow which resembles the anemias observed in advanced renal diseases in man. The same picture is shown by rats with kidney damage due to isoguanine, 8-oxyadenine, inorganic mercuric chloride, or by nephrectomized animals. Levels of adenine

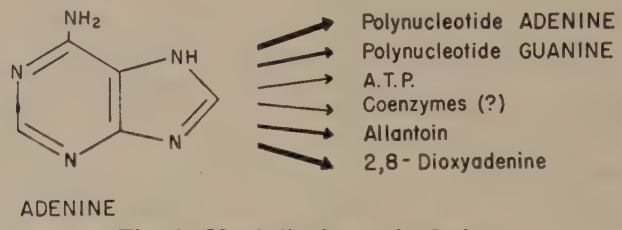

Fig. 8 Metabolic fates of adenine.

or isoguanine which give rise to the kidney damage are considerably above anything which might be considered normal to the animal. Also with both the toxic purines, diaminopurine and adenine, there appears to be a threshold-like relationship between tolerated and toxic doses.

Of the various metabolic fates which adenine may undergo (fig. 8), it would appear that the effects of large doses of adenine are due only to the one pathway, that of the deposition of crystals of 2,8-dioxyadenine, particularly since it is known that intermediates on that pathway will produce an identical pharmacological picture. The toxic levels are from two to 20 times those at which we have administered adenine in other metabolic experiments. It would thus appear that this toxicity of adenine can be related solely to the kidney damage and not to its metabolic role as a precursor of polynucleotide adenine, polynucleotide guanine, ATP, or allantoin.

Pyrimidines occurring in nucleic acids are not utilized by the adult rat when furnished in the diet. This was shown for uracil and thymine in initial studies by Plentl and Schoenheimer ('44) and we have recently shown the same to be true with cytosine (Bendich, Getler, and Brown, '49). Orotic acid, isolated by Mitchell from certain *Neurospora* mutants, was recently studied by the group with Hammarsten. Labeled orotic acid was found (Arvidson, Eliasson, Hammarsten, Reichard, von Ubisch, and Bergstrom, '49) to serve as a precursor of nucleic acid pyrimidines, and this is the first concrete clue concerning the route by which pyrimidines may be elaborated *in vivo*.

In the usual diet of the mammal there would be no more than traces of free purines or of free pyrimidines but there may be considerable amounts of intact nucleic acids. Investigations of the metabolic fate of an intact nucleic acid have indicated that the fates of purine and pyrimidine derivatives are quite different from those of the free bases. We used yeast nucleic acid, prepared from yeast grown in a medium containing isotopic ammonia, and all the nitrogens were essentially equally labeled with isotopic nitrogen.

This intact nucleic acid has been given to rats in their diet and the mixture of mononucleotides derived from it by partial hydrolysis has also been administered intraperitoneally to other rats (Roll, Brown, DiCarlo, and Schultz, '49). It is difficult to interpret these experiments in fine detail since they involve simultaneous administration of derivatives of 4 compounds, each labeled with the same isotope to the same extent. However, two definite conclusions are possible: (1) that the yeast nucleic acid does furnish something quite effective as a pyrimidine precursor; (2) that its purines are utilized much less effectively than are equivalent amounts of free adenine.

When the results from the oral administration are compared with those from a typical experiment with free adenine at only half that molar level (fig. 9), it is evident that the purines of the intact nucleic acid are but very poorly utilized

by the rat for the formation of tissue nucleic acids. In the case of the intraperitoneally administered mixture of mononucleotides the utilization of the purines was considerable but it was still less than that observed with free adenine. This relative ineffectiveness of the orally administered nucleic acid in furnishing a precursor for tissue nucleic acid purines correlates with the presence in the intestine, and to a lesser extent in the tissues, of enzymes which effect the rather complete and rapid degradation of the purine nucleotides.

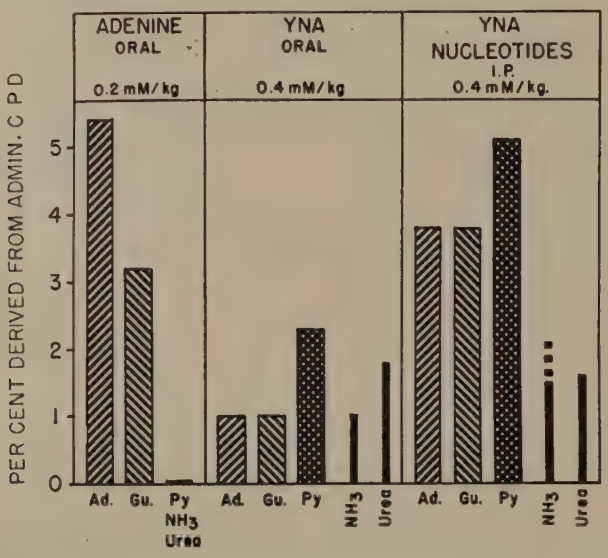

Fig. 9 Incorporation of dietary yeast nucleic acid and nucleotides.

Another point which should be noted is that when the nucleotides were the precursors the amount of isotope in the two purines was equal, whereas the usual ratio is about two to one when adenine is the percursor of both. Whether this may be due to a greater facility of conversion of adenylic acid to guanylic acid, to a direct utilization of the guanylic acid, or to other factors, are questions which remain to be investigated.

From the results it may be seen that appreciable amounts of the tissue nucleic acid pyrimidines were derived from the

nitrogen of either the oral nucleic acid or the mononucleotides. This is in distinct contrast to the results when the free pyrimidines are included in the diet. It implies that complete breakdown to free pyrimidines did not occur and that some larger moiety was utilized by the rat. At almost the same time that we obtained this data, Hammarsten, Reichard, and Saluste ('49, '50) prepared the pyrimidine nucleosides, also through biosynthesis in yeast. Their results with cytidine and uridine showed that cytidine was an effective precursor of both cytosine and uracil of rat nucleic acids, whereas uridine was a relatively ineffective precursor of either.

SUMMARY

In summary, we have a situation where certain free purines are used for the biosynthesis of nucleic acids but their derivatives, as occurring in pentose nucleic acid, are more extensively catabolized and are but poorly utilized for nucleic acid production. In contrast, the pyrimidines, cytosine and uracil, are not utilized for nucleic acid synthesis while ribose derivatives are so utilized.

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We wish to thank Dr. H. M. Kalckar and Dr. W. E. Cohn for information prior to publication regarding the synthesis of adenine-8-C^{14} and the isolation of nucleotides.

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BIOSYNTHESIS OF THE PURINES

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TWO FIGURES

Research on the biosynthesis of the purines has led to the testing of proteins, carbohydrates and their metabolic intermediates as precursors of the excretory products of purine metabolism. Thus alanine, glycine, asparagine, glutamine, and pyruvate (Lewis, Dunn and Doisy, '18; Christman and Mosier, '29; Borsook and Keighley, '35; Quick, '32; Gibson and Doisy, '23) have been shown to increase the excretion of uric acid in the fasting man, whereas lactic and glycolic acids lack this ability (Gibson and Doisy, '23). Fisher ('35) has claimed, however, that L(+) lactate increased the daily output of uric acid by the pigeon. Considerable controversy has risen over the role of histidine and arginine as the precursors of the imidazole nucleus of purines since the report by Ackroyd and Hopkins ('16) that the excretion of allantoin by the rat is lowered when animals were placed on diets deficient in these amino acids. The suggestion of Wiener ('02) that uric acid is synthesized biologically by the condensation of tartronic acid with 2 moles of urea proved an interesting beginning hypothesis which subsequent theories have now replaced.

Investigations on the biosynthesis of purines by the classical feeding techniques are, however, open to the criticism of Lennox ('25) who has shown that the fasting subject gradually increases his stores of blood and tissue uric acid which may be released by the ingestion of certain foodstuffs, notably carbohydrates and proteins or amino acids.

The use of isotopic N^{15} has shown that several substances previously studied as possible precursors of purines are, in fact, not involved. Thus isotopically labeled urea (Barnes and Schoenheimer, '43), histidine (Tesar and Rittenberg, '47), arginine (Block, '46) and pyrimidines (Plentl and Shoenheimer, '44) are not incorporated to a significant extent into either the nucleotides or purine excretory products.

Although it has long been known that the purines may be readily synthesized from dietary ammonium salts (von Knierem, 1877), it remained to Barnes and Shoenheimer ('43) to demonstrate that N^{15}-labeled ammonium salts were incorporated rapidly not only into the purine excretory products but also into the cellular nucleic acids. This paper was largely responsible for calling attention to the fact that purines are synthesized from small carbon and nitrogen units rather than from larger preformed metabolic units of the diet.

Synthesis of purines in vivo. With this thought in mind, investigations on the precursors were undertaken at the University of Pennsylvania laboratory with isotopically labeled derivatives of protein, fat, and carbohydrate metabolism. During the course of the last 4 years the biosyntheses of uric acid, allantoin, adenine, guanine, and hypoxanthine, as well as the pyrimidines have been studied both *in vivo* and *in vitro*. Uric acid was chosen as the initial subject of investigation (Sonne, Buchanan, Delluva, '46; Buchanan and Sonne, '46; Sonne, Buchanan and Delluva, '48; Buchanan, Sonne and Delluva, '48) because it is readily obtained from the excreta of birds and easily degraded by chemical methods into its separate carbon atoms.

The chemical degradation of uric acid was carried out by well-known reactions from the old German chemical literature. Two general procedures were used. The first of these is outlined in the reactions of figure 1 (Edson and Krebs, '36; Schuler and Reindel, '33; Fischer and Ach, 1899; Behrend, '04; Siemonsen, '04). The degradation of uric acid by alkaline manganese dioxide to allantoin resulted in the liberation of carbon 6 of uric acid as carbon dioxide. The further hydrolysis

of allantoin yielded glyoxylic acid in which the carboxyl and aldehyde carbons were derived from what had been carbons 5 and 4, respectively, of uric acid. Glyoxylic acid was concentrated as the lead salt and then converted to the semicarbazone for purposes of purification and identification. The two carbon atoms of glyoxylic acid were readily separated by reacting the semicarbazone of glyoxylic acid with acid permanganate yielding rapidly two carbon dioxide molecules and one formic acid. One of the carbon dioxide molecules is derived from the carboxyl carbon of glyoxylic acid whereas the formic acid comes

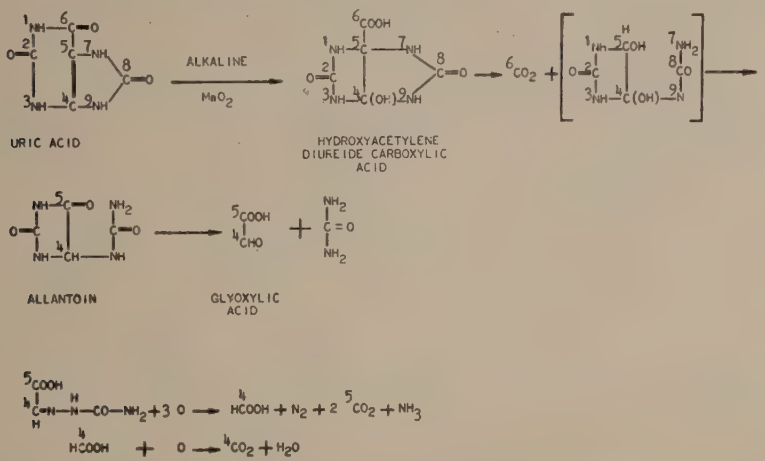


Fig. 1 Reactions of degradation I of uric acid by alkaline manganese dioxide.

from the aldehyde carbon. Formic acid is slowly converted into carbon dioxide thus providing the separation of carbons 4 and 5 of uric acid. The ureide carbon atoms, 2 and 8, are liberated together as urea so that a second degradation procedure of uric acid must be used to separate these two carbon atoms. These are shown in the reactions in figure 2. Uric acid is converted into alloxan by reaction with potassium chlorate in strong hydrochloric acid thereby liberating urea containing what had been carbon 8 of uric acid. By cautious reduction of alloxan by hydrogen sulfide the water-insoluble alloxantin may by obtained. This compound, upon oxidation with lead

dioxide, yields urea which contains what had been carbon 2 of uric acid (Wöhler and Liebig, 1838). All urea samples were converted to carbon dioxide by urease for analysis in the mass spectrometer.

From a metabolic point of view, lactic acid seemed a likely starting point since it occupies a central position in protein and carbohydrate metabolism. The possibility was at first considered that the three-carbon compound, lactic acid, was a direct precursor of the three-carbon chain of uric acid. This initial working hypothesis was readily invalidated, however, by experiments in which either isotopic carbon dioxide, car-

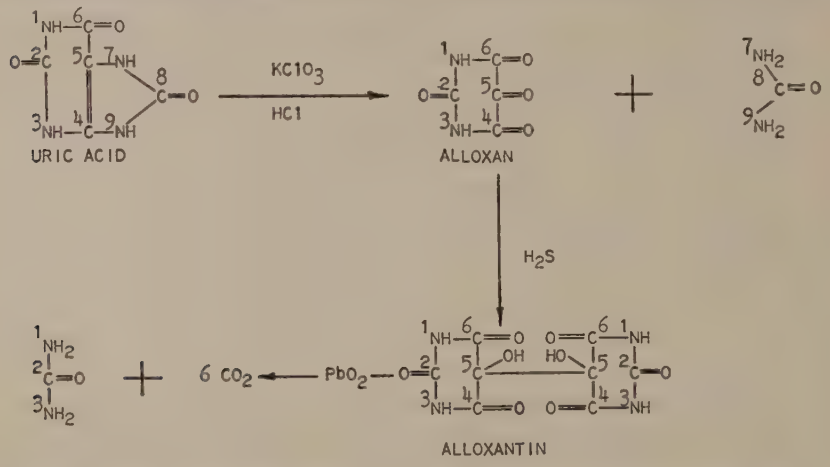


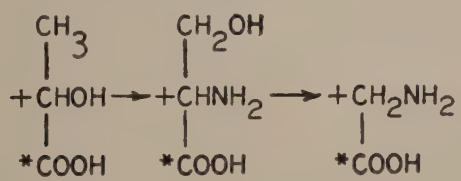
Fig. 2 Reactions of degradation II of uric acid by hydrochloric acid and potassium chlorate.

boxyl-labeled lactate, or α, β-labeled lactate were administered to pigeons and the uric acid of the excreta isolated and degraded.

As may be seen in experiment 1, table 1, carbon 6 of uric acid is derived entirely from carbon dioxide of the animal fluids. The C^{13} concentration of carbon 6 was identical with that of the respiratory gases not only in the first experiment with isotopic bicarbonate but also in all subsequent experiments with isotopically labeled compounds. It was therefore concluded that, after the administration of isotopic organic com-

pounds, the appearance of C^{13} in carbon 6 of uric acid resulted from the oxidation of these compounds to carbon dioxide and the subsequent incorporation of this isotopic carbon dioxide into carbon 6 of uric acid. A somewhat smaller amount of isotopic carbon dioxide was incorporated into carbon 4.

As shown in table 1, experiments 4 and 5, carbon 4 of uric acid may be derived from the carboxyl carbon of lactate, whereas carbon 5 is derived primarily from the α- or β-carbon atoms. It was thus apparent that two of the three carbon atoms of the carbon chain were derived from lactic acid whereas the third was formed from carbon dioxide. It was thought, therefore, that lactic acid was converted into some two-carbon compound prior to its utilization in uric acid synthesis. Fortunately at that time Shemin ('46) was investigating the conversion of serine to glycine in the rat. He had found that the β-carbon of serine is removed so that the carboxyl and α-carbons of serine are the precursors of the carboxyl and α-carbon atoms of glycine respectively. We therefore believed that lactate might be converted to glycine in a similar manner.



(1)

Upon testing carboxyl-labeled glycine it was found that uric acid was synthesized containing a high concentration of C^{13} in carbon atom 4. Subsequent experiments by Shemin and Rittenberg ('47) demonstrated that nitrogen 7 of uric acid excreted by man is derived from nitrogen of glycine. Karlsson and Barker ('49) have also shown conclusively that carbon atom 5 of uric acid of pigeons is derived from the α-carbon atom of glycine.

In conjunction with the above investigations an attempt was made to ascertain the precursors of the ureide carbons (2 and

TABLE 1

Biological precursors of uric acid in the pigeon

EXPT. NO.	PRECURSOR	RATE GIVEN IN mM PER HOUR	C^{13} CONCENTRATION (ATOM PER CENT EXCESS)						
			Labeled carbons of precursor	Respiratory CO_2	Uric acid carbon number				
					6	5	4	2	8
1	C^*O_2	0.75	8.13	0.28	0.25	0.00	0.07	0.02	0.02
2	HC^*OOH	0.75	3.34	0.01	0.01	. .	. .	2.41	2.41
3	$NH_2CH_2C^*OOH$	0.50	5.20	0.12	0.11	0.14	1.13	0.00	0.00
4	DL-CH_3CHOHC^*OOH	0.50	8.80	0.25	0.26	0.00	0.31	0.01	0.01
5	DL-$C^*H_3C^*HOHCOOH$	0.50	5.40	0.11	0.09	0.14	0.04	0.10	0.10

C* = Carbon atoms labeled with C^{13}.

TABLE 2

Incorporation of formate, glycine, and carbon dioxide into nucleic acid and nucleotide purines of the rat
(Heinrich and Wilson)

COMPOUND GIVEN	TOTAL C^{14} GIVEN PER RAT	RADIOACTIVITY OF NUCLEIC ACID PURINES ISOLATED							RADIOACTIVITY OF FREE NUCLEOTIDE ADENINE
		Guanine						Adenine	
		$C_4 + C_5$	C_4	C_6 (est.)	C_2	C_8	Total		
	cts./min.	*cts./min./mM carbon*							*cts./min./mM carbon*
C^*O_2	648,000	35	. . .	770	10	0	170	130	137
$NH_2CH_2C^*OOH$	143,000	530	1,220	. . .	10	10	300	270	300
HC^*OOH	500,000	0	. . .	. . .	16,400	12,500	5,760	4,550	4,490

C* = Carbon atoms labeled with C^{14}.

8) of uric acid. The experiment with isotopic carbon dioxide demonstrated that the ureide carbons of uric acid were not derived from carbon dioxide, thus indicating that the mechanism of synthesis of this metabolic radical differed considerably from the mechanism of the similar metabolic group, urea, by the rat. Although little was known at that time about the biosynthesis of the imidazole ring, the condensation of a diamine and formic acid to form an imidazole ring is a well-known organic reaction. Thus benzimidazole is readily formed from formic acid and orthophenylenediamine (Wagner and

(2)

Millet, '43). This general organic reaction was therefore taken as a model in our biochemical studies. The validity of this working hypothesis was verified by the administration of isotopically labeled formate. As seen in experiment 2, table 1, formate was incorporated into uric acid in large quantity.

Many of the uncertain aspects of the role of formate as an important intermediate in metabolism have been cleared up since the initial finding of its participation in uric acid synthesis. Karlsson and Barker ('49) as well as Sakami ('48, '49) and Winnick, Moring-Claeson and Greenberg ('48) have shown, for example, that formate may be produced from the α-carbon of glycine during its oxidation and may be utilized in uric acid or serine synthesis. The β-carbon of serine is a major source of formate in the body (Elwyn and Sprinson, '50; Siekevitz and Greenberg, '50). The α-carbon of lactate is in all probability a source of formate in so far as it may be converted into glycine by the reactions of equation 1 (Buchanan, Sonne and Delluva, '48; Anker, '48). The methyl groups of choline, methionine, and acetone may all be oxidatively converted into formate (Sakami, '49, '50; Siekevitz and Greenberg, '50). Some of the above reactions are reversible since it has been shown that formate is utilized both in the synthesis

of serine (Sakami, '48) and the methyl groups of methionine and choline (Welch and Sakami, '50).

Formate is oxidized at a very slow rate by the pigeon (Sonne, Buchanan and Delluva, '48) but fairly rapidly by the rat (Sakami, '48; Marsh, '48). The mechanism of formate oxidation has not yet been studied, however.

In initial experiments (Sonne, Buchanan and Delluva, '48) it was reported that the carboxyl carbon of acetate was also a major precursor of the ureide carbons of uric acid. Elwyn and Sprinson ('50), however, could not repeat this result. A reinvestigation of this problem has shown that the acetate used in the first experiments was contaminated with formic acid, and that the apparent utilization of acetate in this experiment was, in fact, due to the presence of a formic acid contaminant (Schulman, Buchanan and Miller, '50). The acetate used in the original experiments was prepared from dimethyl sulfate and sodium cyanide. The production of formate as a by-product of this reaction may be avoided, provided the acidity of the dimethyl sulfate is eliminated by redistillation.

The problem of the precursors of nucleic acid and nucleotide purines has now been substantially explored both in yeast and in the animal. Heinrich and Wilson ('50) have shown that formic acid, glycine, and carbon dioxide all contribute in major proportions to the synthesis of adenine and guanine of nucleic acids of the rat tissue. The results of their experiments are tabulated in table 2.

The guanine was degraded by two procedures which permitted either the direct determination of the C^{14} specific activity of some of the individual carbon atoms of guanine or an indirect computation of these values.

In the first procedure, guanine was oxidized by acid permanganate to guanidine (carbon 2), urea (carbon 8) and carbon dioxide. Guanidine was isolated as the picrate while urea was converted to carbon dioxide by the action of urease. The specific activity of carbon 2 of guanine could be obtained accurately from the radioactive measurements of guanidine picrate. The specific activity of carbon 8 of guanine was not as

TABLE 3

Incorporation of formate and glycine into nucleic acid and nucleotide purines of yeast
(Edmonds and Wilson)

ISOTOPIC COMPOUND USED	DURATION OF EXPT.	TOTAL C^{14} INCUBATED WITH YEAST	RADIOACTIVITY						
			Nucleic acid purines						Free nucleotide adenine
			Guanine					Adenine	
			$C_4 + C_5$	C_4	C_5	C_2	Total		
	hrs.	*cts./min.*	*cts./min./mM carbon*						
$NH_2C^*H_2COOH$	12	2,100,000	4,560	0	9,125	3,700	3,380	3,100	8,650
HC^*OOH	2	700,000	50	..	....	330	130	130	1,510

* Designates carbon atoms labeled with C^{14}.

TABLE 4

Formation of hypoxanthine from glycine and formate in pigeon-liver homogenates

All carboxyl radioactive materials contained 1,000 cts./min./μM. 92 μM of hypoxanthine (HX) added as carrier at conclusion of experiment. Values calculated per gram liver wet weight

EXPT.	COMPOUNDS ADDED	QUANTITY PER FLASK	TOTAL COUNTS IN HX	C^{14} COMPOUND USED IN HX SYNTHESIS	HX FORMED FROM C^{14} COMPOUNDS	C^{14} IN CO_2
		μM	*cts./min.*	μM	μM	μM
1	$NH_2CH_2C^*OOH$	6.8	610	0.61	0.61	1.5
	$HCOOH$	18.8				
2	NH_2CH_2COOH	6.8	1,800	1.80	0.90	0.8
	HC^*OOH	18.8				

* Designates radioactive carbon atoms of isotopic compounds.

accurately measured, however, since urea may be formed from carbon atoms other than carbon 8 of guanine.

In the second procedure guanine was hydrolyzed with concentrated hydrochloric acid yielding glycine (Wulff, 1893). This glycine, isolated as the copper salt, is derived from carbon atoms 4 and 5 of the original guanine (Cavalieri, Tinker and Brown, '49). Upon reacting glycine with ninhydrin, carbon dioxide was evolved from the carboxyl carbon of glycine. Radioactive measurements of this fraction gave the specific activity of carbon 4. From these data the specific activity of carbon 5 of guanine could be calculated. The specific activity of carbon 6 could be estimated at times from knowledge of the specific activity of the entire guanine molecule and the specific activity of the determined values of the other four carbon atoms.

A similar series of experiments have been carried out by Edmonds, Delluva and Wilson ('50) using yeast. The results of these experiments are included in table 3.

The results of experiments in both yeast and the mammal demonstrate that glycine is the precursor of carbons 4 and 5 of the purine structure, whereas formic acid contributes to carbons 2 and 8, and carbon dioxide is the precursor of carbon 6. The general mechanism of synthesis of the purine skeleton is thus identical in the pigeon, rat, and yeast, regardless of the purine studied.

Hammarsten and his associates (Bergstrand, Eliasson, Hammarsten, Norberg, Reichard and von Ubisch, '48), have likewise shown that the nitrogen of glycine is utilized by the rat for adenine and guanine synthesis of the nucleic acids of both the ribose and desoxyribose types. Likewise, the nitrogen of glycine is incorporated into the nitrogen of adenine when incubated with yeast (Abrams, Hammarsten and Shemin, '48).

An interesting comparison may be made of the turnover of nucleic acid and nucleotide purines in the mammal system and yeast. Whereas the turnover of these purine structures from such elementary precursors as glycine, formate, and carbon dioxide is almost identical in rat tissues (Heinrich and Wilson,

'50), Edmonds, Delluva and Wilson ('50) have shown that the turnover of nucleotide adenine is from 3 to 12 times greater than that of nucleic acid adenine in yeast.

Another observation of interest comes from the comparison of specific activities of guanine and adenine after the administration of isotopic precursors. In almost all cases the C^{14} or N^{15} concentration of the purine skeleton of guanine was higher than that of adenine when these purines were derived from isotopic formic acid, carbon dioxide, or glycine. When N^{15}-labeled adenine was administered to rats by Brown, Roll, Plentl and Cavalieri ('48), the N^{15} concentration of the nucleic acid adenine contained significantly more N^{15} than the nucleic acid guanine. Moreover, N^{15} adenine was not incorporated into nucleotide adenine when fed in lower concentrations. Although importantly involved in purine studies, adenine probably does not participate as a direct intermediate in nucleic acid and nucleotide synthesis from lower precursors.

The rat likewise utilizes glycine in the synthesis of its purine excretory product, allantoin. If the C^{14} concentration of allantoin is followed for a period of 10 days during which isotopic glycine is incorporated in the diet, the C^{14} of the allantoin rises and by the 10th day is approximately equal to the average carcass nucleic acid purines isolated at that time (Valentine, Gurin and Wilson, '48). It would thus appear that allantoin results primarily from the breakdown of tissue nucleic acids.

In vitro studies of purine synthesis. Some progress has been made in the understanding of the *in vitro* synthesis of purines by pigeon liver tissue. The synthesis of uric acid *in vitro* by pigeon tissue has certain advantages due to the fact that an intermediate of the reaction, hypoxanthine, accumulates when purine precursors are incubated with pigeon liver slices alone. The accumulation of hypoxanthine results from the fact that pigeon liver does not contain xanthine oxidase. The complete synthesis of uric acid from its precursors may be demonstrated when xanthine oxidase present in other tissues or in milk is added to pigeon liver slices (Edson, Krebs and Model, '36).

Several substances, notably glutamine, oxalacetate, pyruvate, and ammonia stimulate hypoxanthine synthesis although the mode of action of this stimulation is unknown (Örström, Örström and Krebs, '39).

The ammonia-binding mechanism of liver slices studied by Edson, Krebs and Model ('36) is destroyed by destruction of the cell. The study of purine synthesis is further complicated by the fact that after rupture of the cells there is a rapid endogenous breakdown of tissue nucleic acids and nucleotides to form purine bases. Richert, Edwards and Westerfeld ('50) have shown that in liver homogenates of species of animals containing xanthine oxidase up to one-half the initial respiration of the tissue may result from the oxidation of purine bases to uric acid and further products of purine metabolism. In view of this large endogenous breakdown of purine structures in broken-cell preparations it would be difficult to study the new synthesis of the purine skeleton without the aid of isotopic tracers. G. R. Greenberg ('48) of Western Reserve University was able to demonstrate the utilization of both isotopic formate and carbon dioxide in hypoxanthine formation by pigeon-liver homogenates. Further experiments by the Pennsylvania investigators have shown that isotopic glycine is also readily incorporated into newly formed hypoxanthine by this system (Schulman, Buchanan and Miller, '50). When isotopic formate is incubated in a bicarbonate-phosphate medium, the addition of nonisotopic glycine stimulates the incorporation of isotopic formate into the purine skeleton. Vice versa, the addition of formate may stimulate the utilization of isotopic glycine for purine synthesis. This latter effect is particularly noticeable when the homogenate is permitted to age at 0°C. for some few hours. The activity of the systems as measured by the incorporation of isotopic glycine into hypoxanthine is considerably reduced by this procedure and may often be completely restored by the addition of nonisotopic formate (Schulman and Buchanan, unpublished experiments).

In the experiment shown in table 4 an attempt was made to ascertain the relative amounts of glycine and formate utilized

in purine synthesis. When both glycine and formate were incubated with pigeon-liver homogenates so that one compound was isotopic in one vessel and the other isotopic in the other, it was possible to calculate the quantities of isotopic substances utilized for hypoxanthine synthesis when the conditions of incubation were made as nearly identical as possible. As shown in table 4, 1.8 μM of formate were utilized per gram of wet tissue for hypoxanthine synthesis as compared to 0.6 μM of glycine. This ratio of 3:1 approaches as nearly as could be expected the ratio of 2:1 in view of the fact that consideration was not taken of possible dilution of isotopic substrates by non-isotopic materials in the pigeon homogenate (Schulman, Buchanan and Miller, '50).

An interesting and important development in the mechanism of purine synthesis has been reported by Greenberg ('50). He has shown that the addition of phosphorlyated derivatives of ribose stimulates the synthesis of hypoxanthine from isotopic formate. Moreover, in experiments in which inosinic acid, inosine, and hypoxanthine were isolated by paper-chromatographic techniques from homogenates which had been incubated with isotopic formate for varying lengths of time, the specific activities of these three compounds changed in such a manner as to indicate that inosinic acid is an intermediate in the synthesis of hypoxanthine from isotopic formate. Greenberg's experiments, therefore, indicate that ribotide formation is required at one link of the reactions of purine base synthesis.

To date, practically nothing of a conclusive nature has been reported concerning the mechanism of ammonia utilization during purine synthesis by liver homogenates. One step in which formic acid is utilized has been investigated. These experiments will be discussed in the following section.

The possible role of 4-amino,5-imidazolecarboxamide in purine synthesis. The isolation of an amine which accumulates in *Escherichia coli* cultures during sulfa bacteriostasis by Stetten and Fox ('45, cf. Fox, '42) and the subsequent identification of this substance as 4-amino,5-imidazolecarboxamide by Shive, Ackermann, Gordon, Getzendaner and Eakin ('47)

has opened the way to a new compound which may prove of great biochemical interest.

Although, from a structural point of view, 4-amino,5-imidazolecarboxamide seems a promising possibility as a purine intermediate, there are some aspects of its metabolic behavior in bacterial systems which at first glance might argue against its importance. Thus *E. coli* which produces this substance in the presence of sulfa drugs will not utilize it in their absence (Gots, '50). Although this seemed at first disappointing it has now been demonstrated that a purine-requiring mutant of *E. coli* may utilize 4-amino,5-imidazolecarboxamide but requires it in much larger quantities than the corresponding purines in order to obtain equivalent growth (Davis, '50). Fries, Bergström and Rottenberg ('49) have also reported a purine-requiring mutant of *Ophiostoma* which likewise may utilize 4-amino,5-imidazolecarboxamide for growth.

The possibility that the carboxamide accumulates as an abnormal product of sulfa bacteriostasis has now been dispelled by the fact that Woolley and Pringle ('50) have demonstrated that the presence of 4-aminopteroylglutamic acid in the medium may cause the accumulation of the carboxamide compound by *E. coli.*

Likewise, a mutant of *E. coli* has been produced which normally accumulates the carboxamide in the absence of sulfa drugs or other metabolic inhibitors (Gots, '50). Recent experiments with yeast (Schulman and Buchanan, unpublished data) have shown that there is an appreciable and linear utilization of 4-amino,5-imidazolecarboxamide by this system during a two-hour experimental period. Experiments are in progress with isotopic compounds to ascertain whether this metabolism of the carboxamide compound may be accounted for by purine synthetic reactions.

The fact that the addition of glycine to the sulfa-containing culture medium of *E. coli* stimulates considerably the accumulation of 4-amino,5-imidazolecarboxamide provides another interesting link between this compound and the purines (Ravel, Eakin and Shive, '48).

The investigation of this compound was undertaken in our laboratory, not with the point of view that it is per se an intermediate of purine synthesis but with the hope that it could be converted to purine derivatives through enzymatic reactions, part of which involved the true steps of purine synthesis from glycine. For these studies the carboxamide compound was synthesized by Miller and Gurin so that it contained C^{14} in the carbon atom of the 4 position of the imidazole ring. When this isotopic compound was incubated with pigeon-liver homogenates, hypoxanthine could be isolated by a combination of metal precipitation and chromatographic techniques containing quantities of C^{14} which indicated that purine formation

TABLE 5

4-Amino,5-imidazolecarboxamide utilization and hypoxanthine formation by pigeon-liver homogenates

Additions to medium; carboxamide = 3.73 μM = 1,716 cts./min.; 12.5 μM formate; 15.6 μM α-ketoglutarate. Values expressed per gram tissue wet weight

CARBOXAMIDE DISAPPEARANCE		TOTAL [a] HYPOXANTHINE FORMATION FROM CARBOXAMIDE		$\frac{\text{CARBOXAMIDE DISAPPEARANCE}}{C^{14}\text{-HYPOXANTHINE FORMATION}} \times 100$	C^{14} IN RESPIRATORY CO_2
μM	*cts./min.*	*μM*	*cts./min.*	*%*	*cts./min.*
1.13	520	0.78	351	68	2
0.67	309	0.55	254	83	0

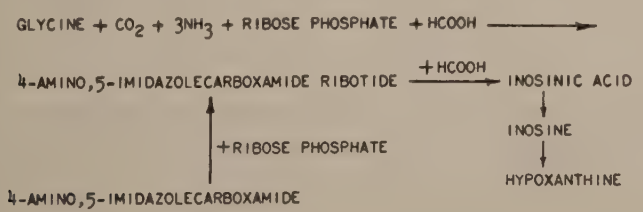
[a] Calculations include added hypoxanthine carrier plus endogenous formation by tissue.

was a major product of carboxamide metabolism in pigeon-liver homogenates (Schulman, Buchanan and Miller, '50). As shown in table 5, between 63 and 82% of the isotopic carboxamide which disappeared could be recovered as isotopic hypoxanthine. Moreover, the order of magnitude of hypoxanthine formation from the carboxamide compound compares very favorably with its rate of formation from formate and glycine. It is also believed that the carboxamide does not break down to glycine prior to its conversion to hypoxanthine since the latter is rapidly oxidized to carbon dioxide in pigeon-liver homogenates (table 4) whereas the carboxamide is not oxidized in this system to any measurable extent (table 5).

In order to clarify the mechanism of the reaction prior to any attempt to isolate enzymes involved in the conversion of 4-amino,5-imidazolecarboxamide to hypoxanthine, a few exploratory experiments were carried out in this laboratory to determine whether the carboxamide is an intermediate in hypoxanthine synthesis from glycine (Schulman and Buchanan, unpublished experiments). Carboxyl-labeled glycine was incubated with pigeon-liver homogenates and a bank of non-isotopic carboxamide. At the conclusion of the experiment the isolated carboxamide contained little or no C^{14}, whereas the hypoxanthine isolated after the addition of carrier contained large amounts of isotopic tracer. This experiment is good but not conclusive evidence that the carboxamide per se is not involved as a direct intermediate in the purine synthesizing reactions. Although the precedure of interposing a bank of a suspected intermediate of metabolism in the reaction mixture has met with considerable success as a tool of intermediary metabolism, there are instances where evidence of this type is negative while other evidence points strongly that the compound in question is indeed an intermediate of the reaction scheme. For example, evidence is available from several independent sources indicating that citric acid is an intermediate of acetate and pyruvate oxidation (Ogston, '48; Potter and Heidelberger, '49; Rudney, Lorber, Utter and Cook, '50; Stern and Ochoa, '49) although attempts to demonstrate this fact by trapping isotope arising from these isotopic substances in added banks of citrate have been unsuccessful (Evans and Slotin, '41; Weinhouse, Medes, Floyd and Noda, '45). Thus, although citric acid is importantly involved in oxidation reactions, it is not possible to demonstrate this relationship with the classical type of "bank" experiment. The negative experiments with 4-amino,5-imidazolecarboxamide of this type are, therefore, interpreted with the above reservation in mind.

Another alternate interpretation is that the carboxamide and glycine are each enzymically converted to a common intermediate of purine synthetic reactions prior to their conversion to hypoxanthine. The above cited experiments of

Greenberg have led to the conclusion that ribotide compounds are involved in the synthesis of hypoxanthine from glycine. The possibility is being examined, therefore, that inosinic acid is an intermediate in the conversion of the carboxamide to hypoxanthine. Thus, experiments were devised in which inosinic acid and hypoxanthine (or inosine) were incubated with radioactive carboxamide for short periods of time. If the hypoxanthine were formed from carboxamide directly without prior involvement of ribose compounds the isolated hypoxanthine should contain a greater specific activity than the inosinic acid. Such, however, was not the case. Under the conditions of our experiments the specific activity of the inosinic acid was 4 to 5 times greater than that of the inosine or hypoxanthine indicating that both the elements of ribose and phosphate are added to the carboxamide prior to ring closure with formic acid. These experiments raise the possibility that 4-amino,5-imidazolecarboxamide ribotide is an intermediate in hypoxanthine synthesis from the carboxamide and perhaps a key metabolite in the biological formation of purines from their more elementary precursors.



(3)

Experiments by Miller, Gurin and Wilson ('50) have shown conclusively that adenine and guanine of the nucleic acids and the muscle adenylic acid of rats may be derived in large measure from injected 4-amino,5-imidazolecarboxamide. In these experiments, C^{14}-carboxamide was administered over a period of three days to rats. The concentration of C^{14} in the above tissue constituents after this time is shown in table 6. A comparison is made between these data on the utilization of the carboxamide compound and the data of Barnes and Schoen-

heimer ('43) on the incorporation of isotopic ammonia into purine structures of tissues. It is assumed that the N^{15} concentration of the urea is a measure of the N^{15} concentration of the available nitrogenous metabolic pool. It would seem that during the three-day period approximately one-twelfth of the purine nucleic acids had turned over, since the concentration of N^{15} of the mixed organs was approximately one-twelfth that of the urea. In the experiments of Miller et al., the C^{14} concentration of the nucleic acid purines of the entire carcass was

TABLE 6

Comparison of the utilization of C^{14}-labeled 4-amino,5-imidazolecarboxamide and N^{15} ammonium salts for the synthesis of purine compounds of the rat

Duration of experiments, 3 days

	COMPOUNDS	SPECIFIC [a] ACTIVITY	ATOM PER CENT [b] EXCESS N^{15}
		cts./min./mM	
1.	4-Amino,5-imidazolecarboxamide administered	412,000	
2.	Carcass purines		
	Nucleic acid guanine	39,200	
	Nucleic acid adenine	48,800	
	Nucleotide adenine	44,400	
3.	Mixed purines of internal organs		1.1
4.	Allantoin	76,200	1.3
5.	Urea		13.0

[a] Data of Miller, Gurin and Wilson ('50). Four hundred milligrams of carboxamide administered per kilogram rat per day.

[b] Data of Barnes and Schoenheimer ('43). Forty milligrams of ammonium citrate (N^{15} = 4.51 atoms per cent excess) administered per kilogram rat per day. Results tabulated in terms of 100 atom per cent excess compound given.

one-tenth that of the injected carboxamide. In all probability the isotopic concentration of nucleic acids of the internal organs was much higher than the average of the carcass since it is known that the turnover of nucleic acid structures of muscle is much less than that of the internal organs, particularly liver. It is thus apparent that the nucleotide formation from carboxamide *in vivo* proceeds at a significant rate.

Since Getler, Roll, Tinker and Brown ('49) have shown that injected hypoxanthine may not be converted to nucleic acids to

a significant extent, the conversion of 4-amino,5-imidazolecarboxamide to nucleic acids *in vivo* does not take place by way of hypoxanthine as an intermediary compound. This statement is made with the assumption that injected hypoxanthine would be handled by the body in an identical fashion with hypoxanthine produced metabolically within the cells.

The administration of isotopic carboxamide results in the excretion of isotopic uric acid by the pigeon (Schulman, Buchanan and Miller, '50) and isotopic allantoin by the rat (Miller, Gurin and Wilson, '50).

In view of the preceding experiments, it would seem that 4-amino,5-imidazolecarboxamide may play an important although not entirely understood role in the reactions of purine synthesis.

BIOSYNTHESIS OF THE PYRIMIDINES

The perplexing problem of the inability of the pyrimidine bases to be incorporated into the pyrimidine nucleic acids of tissue (Plentl and Schoenheimer, '44; Bendich, Getler and Brown '49) has been further studied by the administration of isotopic cytidine and uridine (Hammarsten, Reichard and Saluste, '49) or isotopic yeast nucleic acids (Roll, Brown, Di Carlo and Schultz, '49) to rats and the finding that these substances are incorporated into the pyrimidine nucleotides of nucleic acids. Apparently the inability of the rat to utilize pyrimidine bases lies in the fact that they are not readily converted to the nucleoside form. Arvidson, Eliasson, Hammarsten, Reichard and Bergström ('49) have made the interesting observation that orotic acid, uracil-4-carboxylic acid, is rapidly utilized for the synthesis of pyrimidine nucleic acids in contrast to analogous pyrimidine bases. They have suggested that the presence of the carboxylic acid group in the 4-position of orotic acid permits the formation of the ribotide linkage. These mammalian experiments should be compared to those of Mitchell, Houlahan and Nyc ('48) using *Neurospora.* Experiments are at present underway in several laboratories to ascertain the role of orotic acid in the synthesis of pyrimidine

nucleotides from their elementary precursors. Weed, Edmonds and Wilson ('50) have shown that orotic acid may be incorporated into the pyrimidine nucleotides of the nucleic acids when incubated aerobically with rat liver slices.

Experiments on the elementary precursors of the pyrimidines have led to some interesting findings. Heinrich and Wilson ('50) have shown that carbon 2 of uracil is derived from carbon dioxide. Formic acid does not participate directly in this reaction. Although the precursors of carbon atoms 4, 5, and 6 are not at present known, it has been shown that glycine and carbon dioxide are not involved as precursors of these carbon atoms in the pyrimidines as they are in the synthesis of purine structures. The incorporation of carbon dioxide into carbon 6 of the purine structure and into carbon 2 of the pyrimidine molecules represents in all probability new carbon dioxide fixation reactions.

The fact that pyrimidines and purines, substances of very similar structure, have different precursors and are therefore probably synthesized by entirely different mechanisms has proved to be one of the most interesting developments of the isotopic work on this class of substances.

OPEN DISCUSSION

Chairman HITCHINGS: These two very stimulating papers of Dr. Buchanan and Dr. Brown are now open for discussion. I think we might start back with the *in vitro* work. I wonder if Dr. Greenberg has anything he would like to say?

DR. GREENBERG: It is interesting that, in the pigeon, hypoxanthine is not converted solely to uric acid. Our recent experiments with pigeon-liver preparations indicate that labeled hypoxanthine is converted rather rapidly into inosinic acid via inosine. Whether or not this is the end of that route, I cannot say.

I would like to elaborate on the role of the ribosides and of the imidazolecarboxamide compounds in purine synthesis. We have found that, in pigeon-liver formic acid, carbon dioxide

and carboxyl-labeled glycine are all incorporated into hypoxanthine. Employing C^{14} formate as a tracer, it has been found that radioactivity is incorporated first into inosine-5-phosphate and thence through inosine to hypoxanthine apparently forming ribose-1-phosphate in the reaction. Then, as was said, the hypoxanthine is reversible to inosinic acid by the same general pattern. The evidence that inosine-5-phosphate is the precursor of hypoxanthine in the *de novo* synthesis by way of the over-all mechanism: precursors → inosinic acid → inosine → hypoxanthine, will be presented at Atlantic City. It is based on specific activity measurements and total balance studies, together with other observations. The dilution of the labeled formic acid in the hypoxanthine is quite great. That is, hypoxanthine, as Dr. Buchanan has pointed out, is also derived from concomitant degradation of nucleotides other than inosinic acid. However the dilution of labeled formate when it is incorporated into inosine-5-phosphate is very slight. Thus 2 moles of formic acid are incorporated into each mole of inosinic acid formed. These data are interpreted to suggest that inosinic acid is the first total purine ring which is synthesized in this system. Since inosine follows rather than precedes inosinic acid, the immediate precursor of this nucleotide must be another nucleotide not having a complete structure. By reference to the known occurrence of 4-amino-5-imidazolcarboxamide as a precedent, it seems quite likely that the precursor in question may be a nucleotide of this imidazole compound.

On the addition of ribose phosphate to the system, under certain circumstances, not always, there is found a very large increase in the rate of synthesis of the purines, sometimes as much as threefold or fourfold increase in the rate. Thus it appears that, in the formation of hypoxanthine, a ribose phosphate compound is catalytic. This would suggest a cycle in which ribose phosphate reacts with some compound which eventually forms a nucleotide, this being converted to inosine-5-phosphate and thence to inosine and finally to hypoxanthine

and ribose phosphate. In the intact animal this hypoxanthine is siphoned off as uric acid.

Now we also have been interested in the aminoimidazolecarboxamide compound since it is a logical intermediate. To test the possibility that it was an intermediate in this reaction, we added a pool of this compound to the reaction mixture at the same time that we added radioactive formic acid, and then isolated the carboxamide by paper chromatography at the end of the reaction time. At the end of the experiment there was more than 80% of the imidazol compound remaining. There was no activity whatsoever in the imidazolecarboxamide, whereas at the same time, there was a large incorporation of the labeled formic acid into both inosinic acid and hypoxanthine. There was no change in the rate of incorporation of C^{14} formate as compared to a reaction mixture to which the compound had not been added. This imidazole compound should have been labeled by C^{14} formate since we know that both carbons 2 and 8 of the purines are labeled. We have concluded that this must not be a direct intermediate, but rather, as Dr. Buchanan has suggested, that its riboside might be involved.

Since we have interpreted our findings to indicate the occurrence of a nucleotide of the imidazolecarboxamide as the immediate precursor of inosinic acid, it is logical that such a nucleotide in turn would have as its precursor the riboside of the imidazolecarboxamide. We have considered that this riboside would not be derived from the free base but that the riboside is formed prior to the closure of the imidazole ring. We are at present attempting to bring about an accumulation of some of these hypothetical compounds.

Dr. Buchanan: In line with Dr. Greenberg's remarks on the catalytic effect of ribose compounds on purine synthesis, I might add that one possible explanation why we have been successful in demonstrating the conversion of the carboxamide into hypoxanthine in contrast to the results of investigators using bacterial systems, is the fact that there is a rapid breakdown of nucleotides and nucleosides in the homogenate system. The breakdown of these substances could be furnishing an

important reactant, namely, ribose phosphate, which might condense with the carboxamide as an initial step in the series of reactions leading to purine synthesis from the carboxamide. If this incidental breakdown of nucleotides does not occur in the bacterial system, one would have a possible explanation why the carboxamide is not metabolized in this system.

Dr. Brown: I was wondering whether anybody had taken inosine or inosinic acid and tried to put adenine in with it. Will any exchange occur? If there is an intermediate which is cycling, it does not have to break down into small fragments and go back together again. There could be a simple interchange reaction taking place.

Dr. Buchanan: I would be inclined to believe that adenylic and guanylic acids are products of inosinic acid metabolism and that the mechanism of synthesis of these former two substances is the same up to this point.

Dr. Brown: Another thing (I am thinking particularly of the recent articles from Wajzer) will the ribose of inosine and ribose-3-phosphate exchange so that the necessary inosine-3-phosphoric acid instead of the 5 would be obtained?

Dr. Greenberg: It is possible, of course, that adenine partially breaks down and is synthesized into nucleic acid purine in this state.

Dr. Brown: That is the reason we are being a little suspicious about the whole molecule. We have two nitrogens pinned down and some information on the carbon 8 in the far corner. We have still to watch that the molecule does not open up somewhere. So far there is no indication that it does.

Chairman Hitchings: Another possibility is that adenine could exchange in the whole nucleic acid.

Dr. Brown: There are too many alternative interpretations possible for us to try to use the observation that adenine replaces twice as much adenine in a nucleic acid as it does guanine as the basis for any argument that the whole molecule does not have to be synthesized at one time.

Whether the whole polynucleotide is synthesized at one time, or whether individual nucleotides or individual purines and

pyrimidines can be slipped in and out, is a series of questions for which it will take some time to accumulate answers.

Dr. Buchanan: Is it not true that the nucleotides of the tissues are not turned over with adenine?

Dr. Brown: We have studied muscle adenosinetriphosphate (ATP) representing muscle adenylic acid; and there, at 0.2 m*M* per kilogram with 6% N^{15}, the dilution factor must have been more than 600. We could not detect the renewal. With a higher level of adenine in the diet, about 1.5 m*M* per kilogram, the dilution factor was about 40, indicating some small renewal. However, where there was approximately 2% renewal of the muscle ATP, there was at the same time 13% renewal of the tissue nucleic acids.

Dr. Buchanan: So one certainly could not say that adenylic acid of the tissues would be the precursor of the tetranucleotides.

Dr. Brown: Not within a three-day period. It might get there slowly, but is not a rapid renewal.

Dr. Greenberg: Don't you think, though, that we have to differentiate between the nucleotides in the muscle and viscera?

Dr. Brown: That is right. That is the reason I specified what we are working on, because the free adenylic acid and ATP in the liver may have an entirely different renewal from that of the muscle adenylic acid.

Dr. Bendich: The question is raised whether or not a nucleic acid might be synthesized by the simple expedient of adenine slipping in and out of the preformed molecule or whether the nucleic acid molecule is synthesized all at once.

I think we have some data which shed some light on this question. Dr. Brown presented the experiment in which the mixed nucleotides were given to rats. You will recall that the N^{15} values of the isolated guanine and adenine were identical, whereas in the experiments where adenine alone was given, the adenine N^{15} values were about twice that of guanine. If adenine, let us say, slips in and out at will at a particular rate, one would not expect to get a nucleic acid with the adenine and

guanine N^{15} content identical, as is the case when nucleotides are given.

I would be inclined to interpret the data obtained from the feeding of nucleotides as indicating that perhaps a nucleic acid can be synthesized by putting together the component nucleotides at one time. But that is not necessarily the only way that nucleic acid can be synthesized, and it would be convenient to interpret the data by imagining that, at least in part, the slippage of a small molecule in and out might occur, and that would imply a partial synthesis as well.

Chairman Hitchings: Is there any other discussion?

Dr. Abrams: In relation to tissue specificity or species specificity, we obtained some rather different results from those of Dr. Brown by incubating labeled adenine and labeled guanine with erythroid rabbit bone-marrow slices *in vitro*. It happens that you can make labeled nucleic acids quite well this way, both DNA and RNA; that guanine is used quite as well as adenine, and that guanine is converted to adenine, and adenine is converted to guanine. These conversions are small compared to the adenine-to-guanine conversion observed in the rat.

Another interesting observation in these experiments where marrow slices were incubated with adenine-8-C^{14} and guanine-8-C^{14} was an interconversion of free purines in the Ringer phosphate medium.

When one incubates with adenine and then isolates purines from the medium, one obtains not only adenine, but also guanine and hypoxanthine. The hypoxanthine had about one-third the specific activity of the labeled adenine; guanine had about one-tenth. If one does it the other way around, incubating with labeled guanine, one finds very little free adenine in the medium, not enough to work up chemically, but there is present hypoxanthine which has a specific activity of about 3% of the initial purine as compared to the 30% when one used adenine.

It would seem as though nucleic acids, which of course are being degraded during all this time as well as being synthe-

sized, liberate hypoxanthine and guanine as breakdown products, so that one obtains a mixture of labeled hypoxanthine from the labeled purines which were added, and unlabeled hypoxanthine from nucleic acid degradation.

You get a much higher synthesis of DNA in this sort of tissue than you do in the rat tissues. The ratio of RNA to DNA is approximately 5 to 1.

Dr. Brown: That would be analogous to the regenerating liver?

Dr. Abrams: Yes, in so far as this is a rapidly proliferating tissue.

Dr. Brown: I might mention some data which Dr. Kalckar presented recently at the Harvey Society. He gave 8-labeled adenine to *Lactobacillus casei*, and there he found that the adenine and the guanine each had the same amount of C^{14} per mole, but it was about half that of the adenine in the medium.

With folic-acid-deficient *L. casei* he found that the nucleic acid purines from the microorganism had about the same activity as that of the medium. Of course, the point is that folic acid is involved in purine synthesis. With folic acid, there is some synthesis of purines from nonisotopic precursors. Without it, there is none.

In *L. casei* there is a conversion of adenine into guanine. With yeast we have also made the observation that this organism will convert adenine into guanine. The ratio of the activity in the two purines is about 2 to 1. Yeast will use adenine very efficiently from the medium.

All species so far tested with adenine have used it, and converted it into guanine.

Dr. Bendich: I think you might be interested in some data that demonstrate that there is a considerable difference in how certain species synthesize nucleic acids from purine precursors. You know, the rat utilizes adenine for the synthesis of both purines and also utilizes 2,6-diaminopurine for the synthesis of guanine. The rat does not use isoguanine for purine synthesis. In contrast, there are some mutants of

Escherichia coli requiring a source of purines for their growth, which will not utilize diaminopurine, but will utilize isoguanine and crotonoside (isoguanine riboside).

Dr. Buchanan, I did not quite understand the difference between the incorporation of carbon dioxide into the 6-carbon of uric acid and the 4-carbon of uric acid. In carbon 6, the value was 0.25 and in carbon 4 it was 0.07. It is only one-third, then, of the carbon 6.

I think that Dr. Buchanan had mentioned that he was inclined to believe that the incorporation into carbon 4 was an indirect process, whereas incorporation into carbon 6 is a direct process. Where does one draw the line in such a case?

Dr. Buchanan: I think you can draw it any place you desire. One guess is about as good as another until the complete picture is drawn. Our interpretation that carbon dioxide fixation into position 6 of uric acid is a direct one whereas the incorporation into position 4 is indirect is based primarily on the results we obtained upon feeding carboxyl-labeled lactate. The carboxyl carbon of lactate is directly utilized as a precursor of carbon 4 but not carbon 6 of uric acid. If nonisotopic lactate is allowed to metabolize in the presence of isotopic carbon dioxide, lactate may be formed containing significant amounts of isotope in the carboxyl position. This fixation of carbon dioxide into lactate may be explained by the operation of the Wood-Werkman reaction accompanied by other equilibrium reactions of the tricarboxylic acid cycle. Since carbon dioxide and the carboxyl carbon of lactate both act as precursors of carbon 4, it may be presumed that carbon dioxide is utilized indirectly through these equilibrium reactions involving lactate and the Wood-Werkman reaction. Since the carboxyl carbon of lactate is not involved as a precursor of carbon 6, it may be assumed by similar reasoning that carbon dioxide fixation in this case is direct and not concerned with the Wood-Werkman reactions or other reactions of the tricarboxylic acid cycle.

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THE ORIGIN AND METABOLISM OF RIBOSE[1]

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FIFTEEN FIGURES

Our interest in the origin of ribose arises from investigations of virus synthesis (Cohen, '47, '48a, b). When *Escherichia coli* are infected with bacteriophage the products of the metabolism of the bacteria are changed. The uninfected growing bacteria synthesize nucleic acids needed for formation of more bacteria with a large preponderance of ribonucleic acid (RNA). When these bacteria are infected with T_2, T_4 and T_6 bacteriophages, the bacteria cease to multiply, although metabolism continues. However, nucleic acid is synthesized by the infected bacteria and consists solely of virus nucleic acid, of the desoxyribose type (DNA). Indeed, essentially all phosphorus entering the infected cell is shunted into the synthesis of DNA. In order to investigate the mechanism of this shunt in nucleic acid formation it is necessary to find out what enzymes are involved, raising the problem of the origin of ribose and desoxyribose in normal organisms. In the last three years we have attacked only the problem of ribose formation.

The literature on both the formation and the metabolism of pentose is not extensive. That on the metabolism of pentoses will be briefly reviewed first, because this report will deal mainly with the question of the origin of the pentoses.

[1] The work described in this paper was conducted in part under a grant-in-aid from The Commonwealth Fund, and in part under an Office of Naval Research Contract N6ori-188, Task Order 1, NR-135–055.

METABOLISM OF PENTOSES

Not too much is known of the metabolism of the pentoses. It was the general experience that yeast or yeast extracts were unable to ferment or oxidize free pentoses. Certain bacteria of the acetic acid group were reported (Hermann and Neuschul, '31) to produce the pentonic acids from D-arabinose or rhamnose. Hayasida ('38) isolated arabonic and xylonic acids formed by a species of *Fusarium* from D-arabinose and D-xylose. Although few bacteria utilize pentoses as major energy and carbon sources in nature, strains may be selected which by growth in a medium containing pentose can metabolize that pentose adaptively. These adaptive enzymes in *E. coli* strains, for example, are specific for the one pentose to which the bacteria have been adapted. D-Ribose, D- or L-arabinose, D-xylose are fermented by adapted strains of *E. coli* with the production of 2 moles of acid per mole of substrate (Cohen '49; Cohen and Raff, '50).

Dickens ('38a, b) investigated the oxidation and the fermentation of pentoses and pentose phosphates by yeast extracts and by hemolyzed red cells. Only the phosphorylated pentoses were attacked. D-Ribose-5-phosphate was much more actively metabolized than D-arabinose-5-phosphate or D-xylose-5-phosphate. By *oxidation* of 1 mole of ribose-5-phosphate, 1 mole of oxygen was consumed and 1 mole of carbon dioxide produced. During *fermentation* of 1 mole of D-ribose-5-phosphate by yeast extracts, 1 mole of carbon dioxide and 1 mole of ethanol were produced, and 1 mole of organic phosphate was hydrolyzed. Evidence was also obtained for the formation of a C_2 compound, possibly ethylene glycol. We have observed that ribose-3-phosphate is not fermented by yeast extracts which yield 1 mole of carbon dioxide per mole of ribose-5-phosphate.

It was reported by Dische ('38) that hemolyzed red cells in the presence of sodium fluoride or bromoacetate broke down adenosine with the probable formation of triose phosphate and glycolaldehyde from the ribose. Similarly, Stephenson and Trim ('38) reported that, concomitant with the deami-

nation of adenosine or inosine by *E. coli,* there was a disappearance of pentose, either aerobically or anaerobically. Fermentation of these nucleosides was immediate and rapid while the action on free ribose was delayed and slow. We know now that this delayed utilization of ribose may have been due to adaptive enzyme formation. It appears probable that the nucleosides gave rise to ribose-1-phosphate which was then further degraded (Kalckar, '45, '47).

Investigations of the mode of action of penicillin by Krampitz and Werkman ('47) disclosed that during endogenous metabolism of *Staphylococcus aureus* there was a breakdown of ribonucleic acid, and the disappearance of pentose. Endogenous oxygen consumption and carbon dioxide and acetic acid production occurred in a ratio of 3:3:1. Added RNA and pyrimidine nucleotides were similarly metabolized, but not DNA, ribose, or ribose-5-phosphate. All these reactions were inhibited by penicillin while there was no inhibition of the metabolism of added glucose or any intermediate in the Meyerhof cycle, nor of triose phosphate dehydrogenase.

These findings suggest that in most cells pentose phosphates are the key intermediates arising from nucleotides and nucleosides. It is possible that adaptation of many bacteria to the utilization of free pentose requires the elaboration of pentokinases, comparable to the transphosphorylating hexokinase and the adaptive enzymes, galactokinase (Trucco, Caputto, Leloir, and Mittelman, '48) and gluconokinase (Cohen and McNair Scott, '50b).

However, in some cells oxidative products of free pentoses are seen to be the pentonic acids. Further oxidation may occur in a stepwise manner to 4-, 3-, and 2-carbon fragments and carbon dioxide, although no evidence exists for these later postulated steps.

The data of Dickens ('38) and Dische ('38) on the fermentative degradation of combined ribose are considerably clarified by the brief note of Racker ('48). He has shown the existence of an enzyme in *E. coli* and yeast which splits ribose-5-phosphate to triose phosphate and some two-carbon frag-

ment. The precise nature of the triose phosphate formed is not known, since Racker analyzed for the formation of dihydroxyacetone phosphate in the presence of isomerase. Nevertheless, it is clear that whatever triose phosphate was formed it could be expected to give rise to a mole of carbon dioxide and ethanol in appropriate systems, as in yeast. Thus the existence of a system generating triose phosphate from ribose-5-phosphate seems likely in yeast and red blood cells, as well as *E. coli*. The additional postulate may be made of a phosphopentomutase establishing the equilibrium of ribose-1-phosphate ⇋ ribose-5-phosphate. A reaction of this type has already been observed for desoxyribose phosphate (Friedkin, Kalckar, and Hoff-Jørgensen, '49; Manson and Lampen, '49).

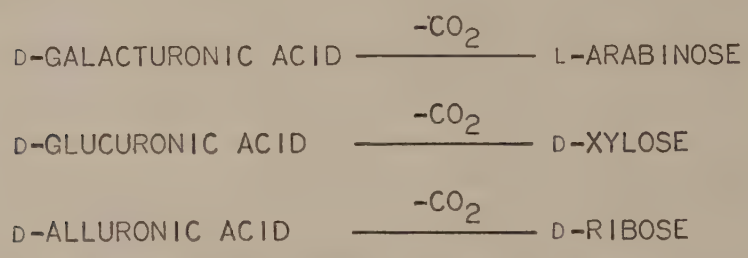


Fig. 1 Hypothesis of pentose formation from uronic acids.

ORIGIN OF PENTOSES

No experiments have ever shown that an organism needs ribose or desoxyribose as ingested metabolites. It would appear that all organisms are able to synthesize both.[2] There have been suggestions of three possible routes of pentose formation. The first suggests the decarboxylation of the corresponding uronic acid, as shown in figure 1. Thus, D-ribose would be obtained from D-alluronic acid, which is not known to occur naturally. The enzymatic decarboxylation of any uronic acid has not yet been shown, although this mechanism

[2] Possible exceptions are suggested by the overcoming of a vitamin B_{12} deficiency in some lactobacilli by any desoxyriboside as well as by the desoxyriboside requirement of *Thermobacterium acidophilus* R_{26}.

has been postulated to account for the existence of a homologous series of a single uronic acid and a single pentose which sometimes may be found in plant gums and mucilages.

In a test of this hypothesis, it was shown that *E. coli* which had been adapted to utilize a uronic acid were not simultaneously adapted to metabolize the homologous pentose (Cohen,

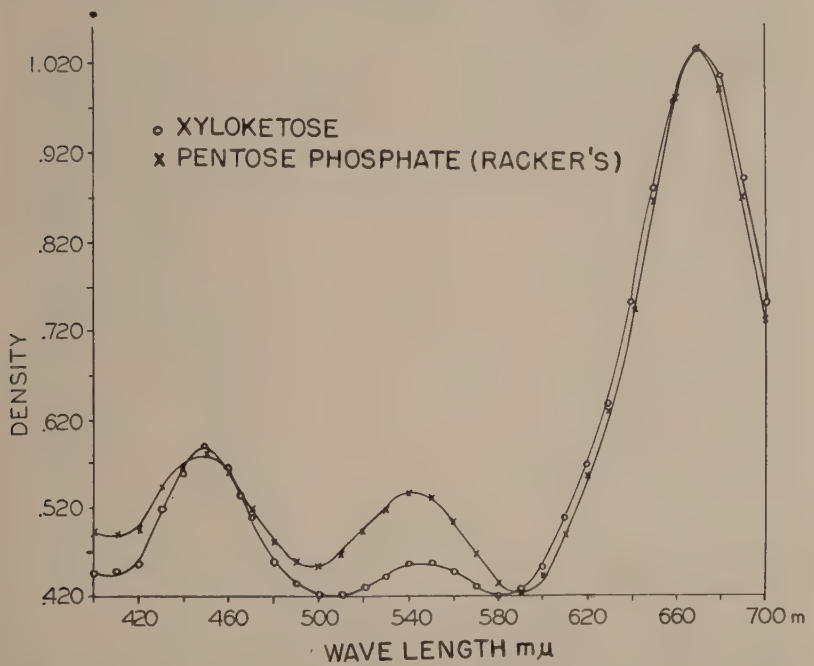


Fig. 2 Absorption spectra of the reaction of various compounds with orcinol-$FeCl_3$ after 40 minutes heating at 100°C. Concentrations are given in micrograms per millimeter of reaction mixture.

a. Racker's phosphate 62 μg/ml. Xyloketose was eluted from a paper chromatogram which had separated a mixture of xylose and xyloketose. The amount was uncertain.

b. D-ribose 4.4 μg/ml
D-xylose 5 μg/ml
D-xylose 2.7 μg/ml
D-arabinose 4.2 μg/ml

c. arabinose-5-phosphate 16.4 μg/ml
ribose-5-phosphate 16.8 μg/ml
xylose-5-phosphate 18.3 μg/ml
ribose nucleic acid 27 μg/ml

d. 2-ketogluconate 20 μg/ml
5-ketogluconate 15.4 μg/ml
glyceraldehyde 10 μg/ml

'49). Thus, *E. coli* adapted to glucuronic acid were not simultaneously adapted to D-xylose or indeed any other pentose. However, if this adaptation involved the acquisition of the ability to phosphorylate glucuronic acid, as does adaptation to gluconate (Cohen and McNair Scott, '50) it is possible

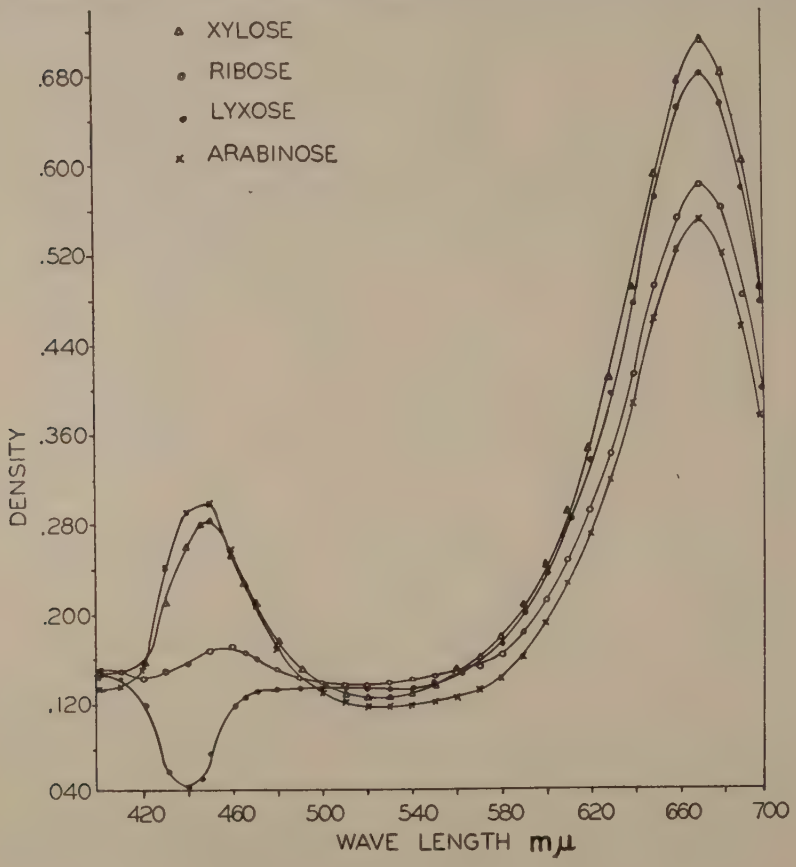


Fig. 2 b See legend under 2 a.

that the phosphoglucuronate would be decarboxylated to yield D-xylose phosphate which, conceivably, could be the intermediate.

The second suggested route postulates the condensation of smaller fragments by an enzyme such as aldolase which

forms fructose-1,6-diphosphate from 2 moles of triose phosphate. Racker ('48) studied the condensation of dihydroxyacetone phosphate and glycolaldehyde by crystalline muscle

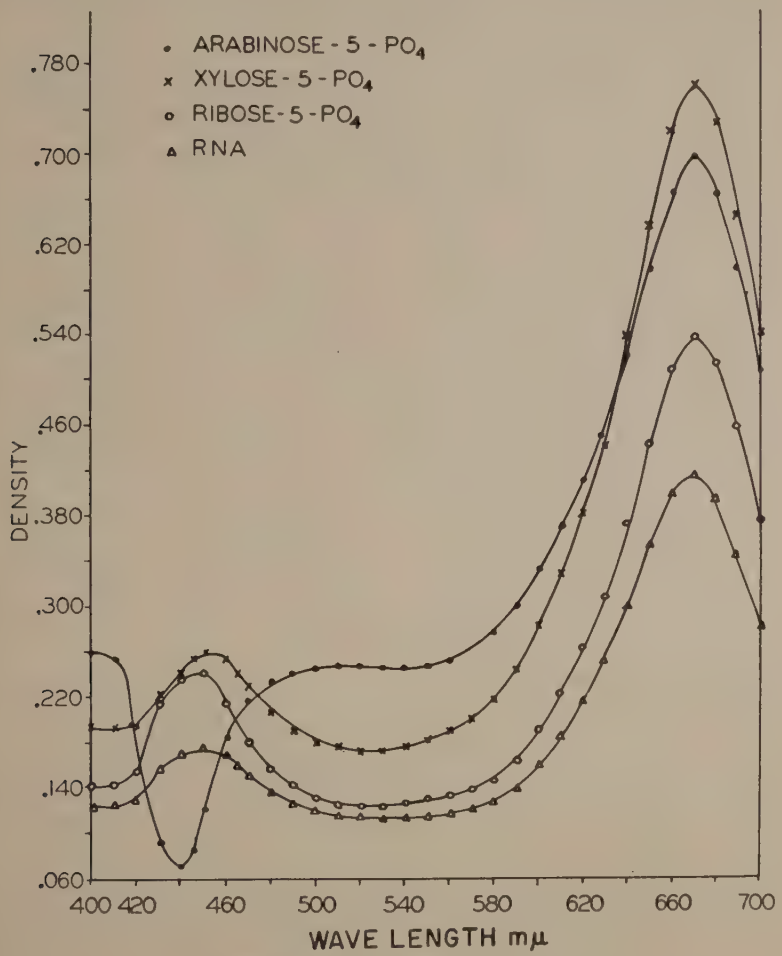


Fig. 2 c See legend under 2 a.

aldolase. However the product appears to be xyloketose-1-phosphate and not an aldopentose. Figure 2 a shows the absorption spectra of the reaction products of orcinol and Fe^{+++} (Bial reaction) with xyloketose and with the phosphory-

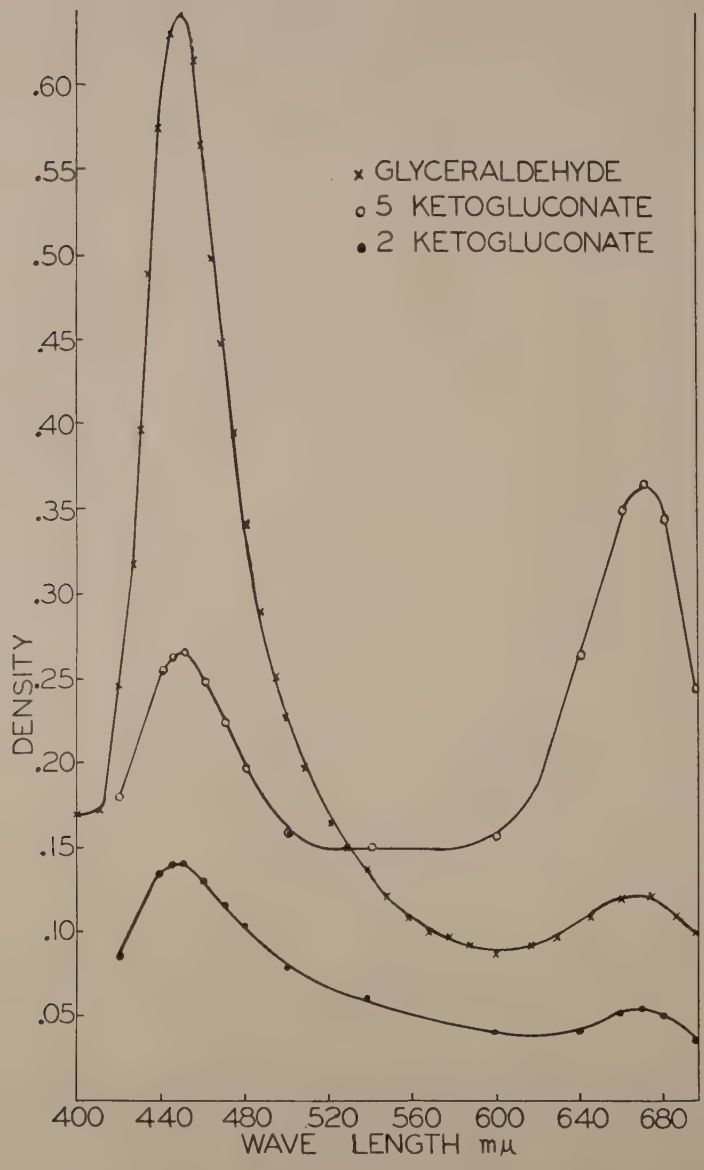


Fig. 2 d See legend under 2 a.

lated product of such an aldol condensation, kindly given us by Dr. Racker. The curves are similar and show a peak at 540 mμ which is not given by any aldopentoses or aldopentose phosphates in the Bial reaction. (See figs. 2 b, c, d.)[3]

Of course, it is not excluded that an enzyme other than the known aldolases may effect an aldol condensation of triose phosphate and a two-carbon fragment to form ribose phosphate. So far attempts at such a condensation have been unsuccessful although such a reaction is implied by the possible reversal of the enzymatic cleavage of ribose-5-phosphate to form triose phosphate, as described by Dische ('38), Dickens ('38b) and Racker ('48). Such a system is under investigation in this laboratory at the present time.

The third possibility, suggested by Lipmann ('36), is that a pentose phosphate might be formed oxidatively from glucose phosphate by way of Warburg's glucose-6-phosphate dehydrogenase (Warburg, Christian, and Griese, '35). In this system glucose-6-phosphate is oxidized to 6-phosphogluconate. Further oxidation of phosphogluconate, according to Lipmann ('36), would yield D-arabinose-5-phosphate. Dickens ('38a) investigated this system and found evidence for the formation of pentose phosphate. He concluded that the pentose formed was possibly ribose-5-phosphate because a yeast extract could both oxidize and ferment ribose-5-phosphate fairly vigorously and was only slightly active with D-arabinose-5-phosphate or D-xylose-5-phosphate. His scheme of the possible course of the reaction is given in figure 3.

Isolation of the products of this reaction was attempted by both Dickens ('38a) and Warburg ('37). By fractional precipitation with barium, mercury, and lead, they obtained salts which appeared by elementary analysis to consist of 3-carbon, 4-carbon, 5-carbon, and 6-carbon phosphates. Some were acids, and some gave strong pentose reactions, and one fraction reacted with resorcinol. Because of the difficulty of preparation of the components of the system, the amounts

[3] We are grateful to Miss Rachel Arbogast and Miss Mary Lanning for aid in the determination of these and other spectra.

of material they had to work with were small and did not allow of further analysis.

Of the three possible routes just described, we have so far obtained positive evidence for ribose formation only by the last oxidative pathway (McNair Scott and Cohen, '49; Cohen and McNair Scott, '50a). Some of the results of investigations of this system will now be discussed.

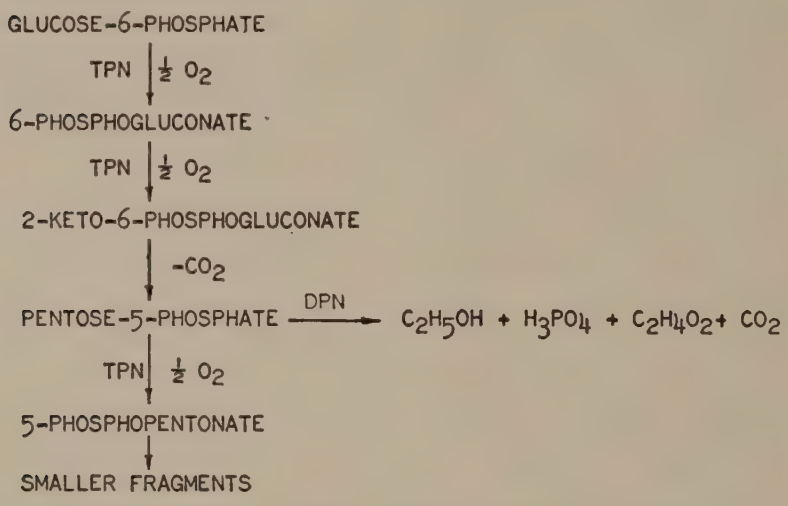


Fig. 3 Dickens' scheme of glucose-6-phosphate oxidation.

PROPERTIES OF THE 6-PHOSPHOGLUCONATE DEHYDROGENASE SYSTEM

The 6-phosphogluconate was allowed to react with triphosphopyridinenucleotide (TPN) in the presence of a suitable enzyme and a carrier to reoxidize the $TPNH_2$. The reaction mixture was then analyzed to detect an increase in pentose. In most of the experiments reported here the enzyme was obtained from a yeast extract by precipitation at pH 4.6 with dilute acetic acid, as described by Dickens and McIlwain ('38).

The specificity of our enzyme preparation is shown in figure 4. One mole of oxygen is consumed during the oxidation of 1 mole of glucose-6-phosphate. The rate of oxidation of

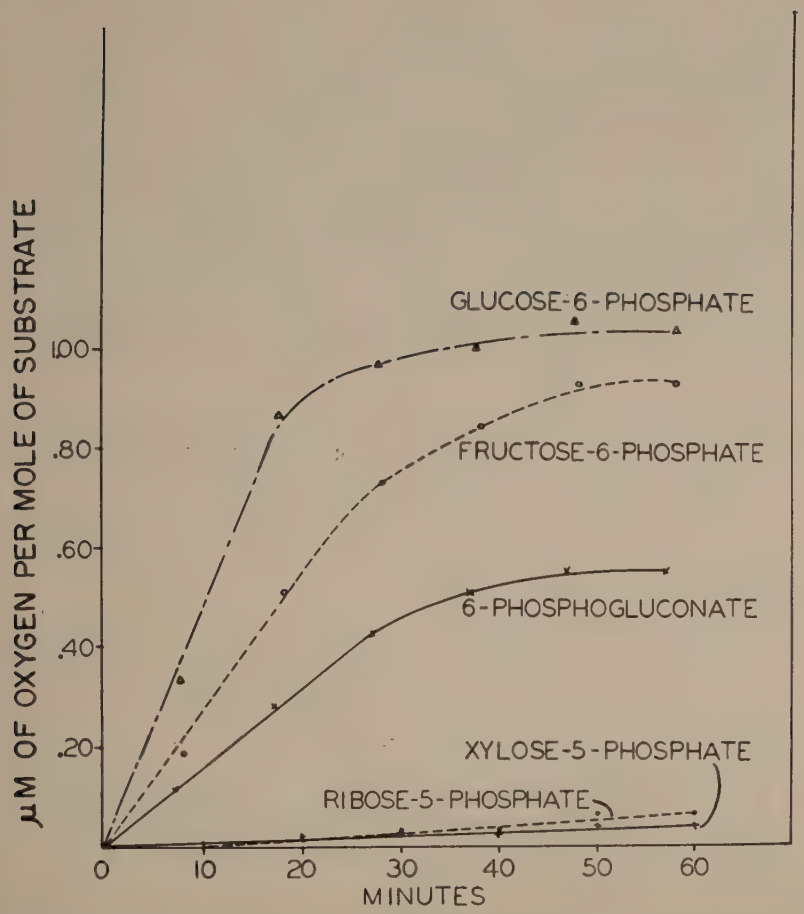


Fig. 4 Substrate specificity of the dehydrogenase. The reaction mixture contained 20 mg enzyme C, 0.1 mg TPN, 55% purity, and substrate in 2 ml of 0.0035 *M* phosphate pH 6.6. One side bulb contained 0.6 mg phenazine methochloride in 0.2 ml, tipped at 0 time. The other side bulb contained 0.2 ml of 0.5 *N* NaOH. The center well contained 0.3 ml of 50% trichloroacetic acid. Incubation was in air at 38°C., for 60 minutes. Substrates were glucose-6-phosphate 5.4 μM, fructose-6-phosphate 7.5 μM, 6-phosphogluconate 11.3 μM, or 10 μM of glucose-1-phosphate, glucose-4-phosphate, glucose, ribose-5-phosphate, ribose-3-phosphate, xylose-5-phosphate or glyceraldehyde-3-phosphate.

No oxygen uptake with glucose-1-phosphate, glucose-4-phosphate, ribose-3-phosphate, glyceraldehyde-3-phosphate, glucose.

fructose-6-phosphate is less than that of glucose-6-phosphate but the total oxygen consumed also approaches 1 mole. With 6-phosphogluconate approximately 0.5 mole of oxygen was consumed. The oxygen consumption with D-ribose-5-phosphate as substrate was less than 0.1 mole in one hour, and even less with D-xylose-5-phosphate. This enzyme showed no activity with other phosphate esters tested, namely glucose-1-phosphate, glucose-4-phosphate, ribose-3-phosphate, glyceraldehyde-3-phosphate, nor with glucose or gluconate. It was found that the enzyme preparation contained some phosphohexoisomerase which probably accounts for the activity on fructose-6-phosphate.

The presence of inorganic phosphate is not necessary in the reaction mixtures and, in fact, at a concentration of 0.02 *M* and higher, oxygen consumption and pentose production are inhibited. As noted by Dickens ('38a), cyanide appeared to be an activator in this system with most preparations of enzyme.

All our earlier experiments were performed using coenzyme prepared from horse liver by Warburg's method ('36b). This yielded small amounts of an impure product. In our most recent work, we have used TPN of 55% purity. The rate and the end point of oxygen consumption are dependent on the TPN concentration at low concentrations. 6-Phosphogluconate was prepared by the bromine oxidation of glucose-6-phosphate and isolated as the barium salt. The carrier used in manometric experiments was phenazine methochloride as suggested by Dickens and McIlwain ('38).

A typical experiment is presented in figure 5. At intervals the reaction was stopped in one vessel by tipping in trichloroacetic acid to precipitate the protein and determine total carbon dioxide. Reducing sugars were determined in the supernatant fluid by a colorimetric method (Nelson, '44) and pentose by the Bial reaction (Miller, Miller, and Golder, '50).

The rate of oxygen consumption decreased as the reaction proceeded and usually became very slow when about 0.5 mole

of oxygen had been taken up per mole of phosphogluconate. Sometimes the ratio was slightly greater than this. Carbon dioxide produced was usually about 1 mole per mole of oxygen consumed. In our most recent experiments carbon dioxide was slightly less and equal to the pentose produced. The pentose produced was usually equivalent to 0.25 to 0.40 moles per mole of phosphogluconate. In the experiment presented in figure 5, starting with 70 mg of phosphogluconate, about 9 mg of apparent pentose, i.e., Bial reactive materials, accumulated as end products of the reaction. It is important to stress that this pentose is not derived from the RNA of the enzyme preparation.

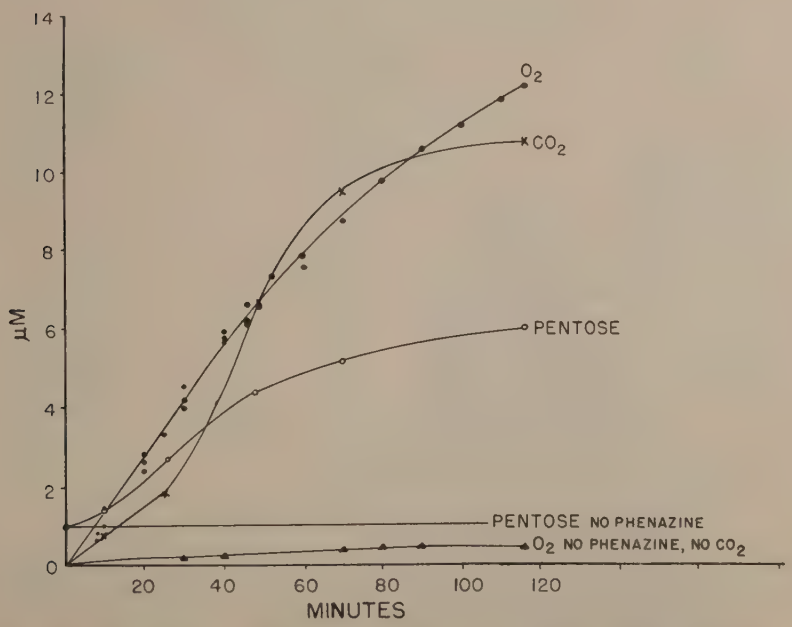


Fig. 5 Gas exchange and pentose accumulation during phosphogluconate degradation. In each vessel were 2 ml enzyme mixtures containing 10 mg enzyme D, 2.34 mg TPN — 3% purity, 23.2 μM phosphogluconate, 0.06 ml NaCN 0.2 M and phosphate buffer 0.003 M of pH 7.4. Sidearm 1 contained 0.6 mg phenazine methochloride in 0.2 ml and was tipped at 0 time. Sidearm 2 contained 0.2 ml 0.5 N NaOH. The well contained 0.2 ml 5% trichloroacetic acid which was tipped at the times indicated to liberate CO_2.

ANALYTICAL METHODS

In order to determine the nature of these substances several types of microanalytical procedures have been adopted.

Paper chromatography of sugars and sugar phosphates has been very useful (Partridge, '46, '48). The reaction mixtures have been chromatogrammed both before and after treatment with alkaline phosphatase (Schmidt and Thannhauser, '43). By this procedure on standard materials, it has been possible to show ribose as the only sugar in the pentose phosphates derived from adenosine-5-phosphate and the aden-

TABLE 1

R_f values for sugar phosphates

COMPOUND	SOLVENTS	
	80% ethanol containing 0.8% acetate at pH 3.5	80% ethanol containing 0.64% boric acid
Glucose-6-phosphate	0.35	0
Fructose-6-phosphate	0.38	0
Glucose-1-phosphate	. .	0
Glucose-4-phosphate	0.45	0
Ribose-5-phosphate	0.50	0
D-Arabinose-5-phosphate	0.54	0.25
D-Xylose-5-phosphate	0.55	0, 0.25
Ribose-3-phosphate	0.50	0, 0.19
Xylose-3-phosphate	0.53	0, 0.23
Glyceraldehyde-3-phosphate	0.73	0.92
	0.83 fluorescence	0.87
6-Phosphogluconate	0.89	. .

osine phosphate derived from RNA, glucose in Embden's ester and Cori ester, and fructose in hexose-1,6-diphosphate.

For chromatography of the phosphates, a one-phase solvent mixture containing ethanol and acetic acid was used. Ethanol-boric acid mixtures have also been used, since by means of the Boeseken reaction, compounds, such as ribose-5-phosphate which contains two *cis*-hydroxyls, could be separated from D-arabinose-5-phosphate or ribose-3-phosphate which do not have this grouping. In table 1 are presented

the R_f values of some sugar phosphates. Positions on the chromatogram were identified by a variety of indicators including an ultraviolet light before and after indicator application, bromphenol blue, Benedict's reagent, m-phenylenediamine, and o-phenylenediamine which is particularly useful for 2-ketoacids (Cohen and Lanning, '50). The papers

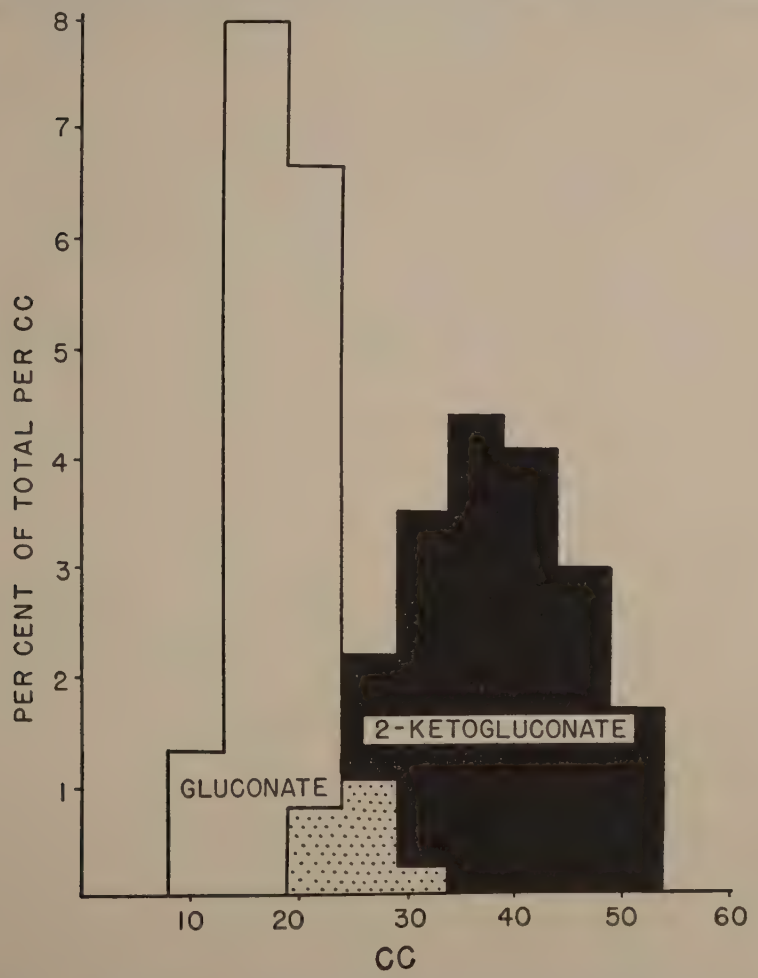


Fig. 6 The elution of about 10-mg quantities of gluconic acid and 2-ketogluconic acid from an anion-exchange column (14 cm) with 0.01 *N* HCl. Flow rate was 0.5 ml per minute.

have been cut and extracts analyzed for pentose, reducing sugar, and phosphate. Recoveries of better than 90% have been obtained in the summated extracts of such cuts.

Ion-exchange columns have given good separation of some components. A mixture of neutral sugars and salts which would include gluconate and 2-ketogluconate were passed through an anion-exchange resin, Amberlite IRA-400. The neutral materials were further deionized by passage through

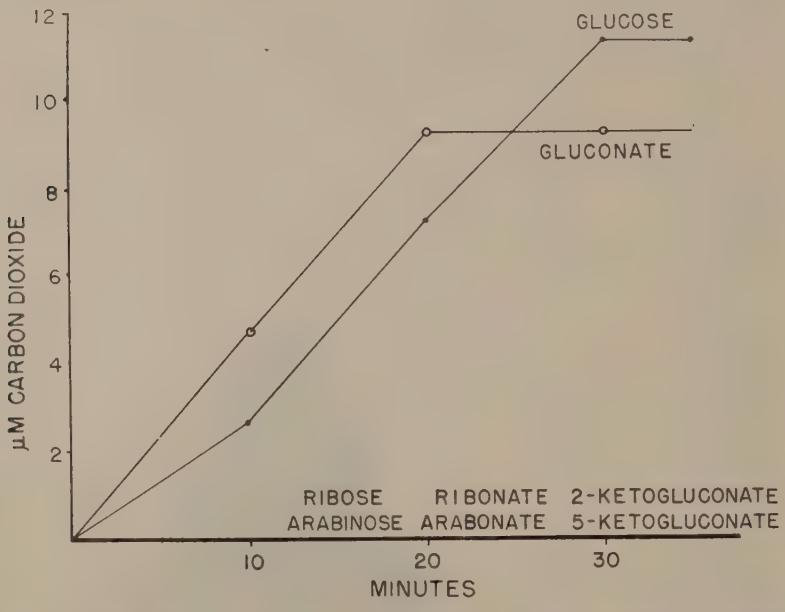


Fig. 7 Fermentation of various substrates by *E. coli* adapted to gluconate.

Amberlite IR-100 and treatment with silver oxide. The removal of inorganic salts is important in obtaining clean chromatograms, as well as for preparation of derivatives. The adsorbed acids on the anion-exchange column were eluted by 0.01 *N* hydrochloric acid at a flow rate of 0.5 ml per minute which thereby effected a separation of gluconate and 2-ketogluconate, as shown in figure 6. The neutral sugars and organic acids have recently been separated by adsorption from alcohol solutions on Florex and elution with different

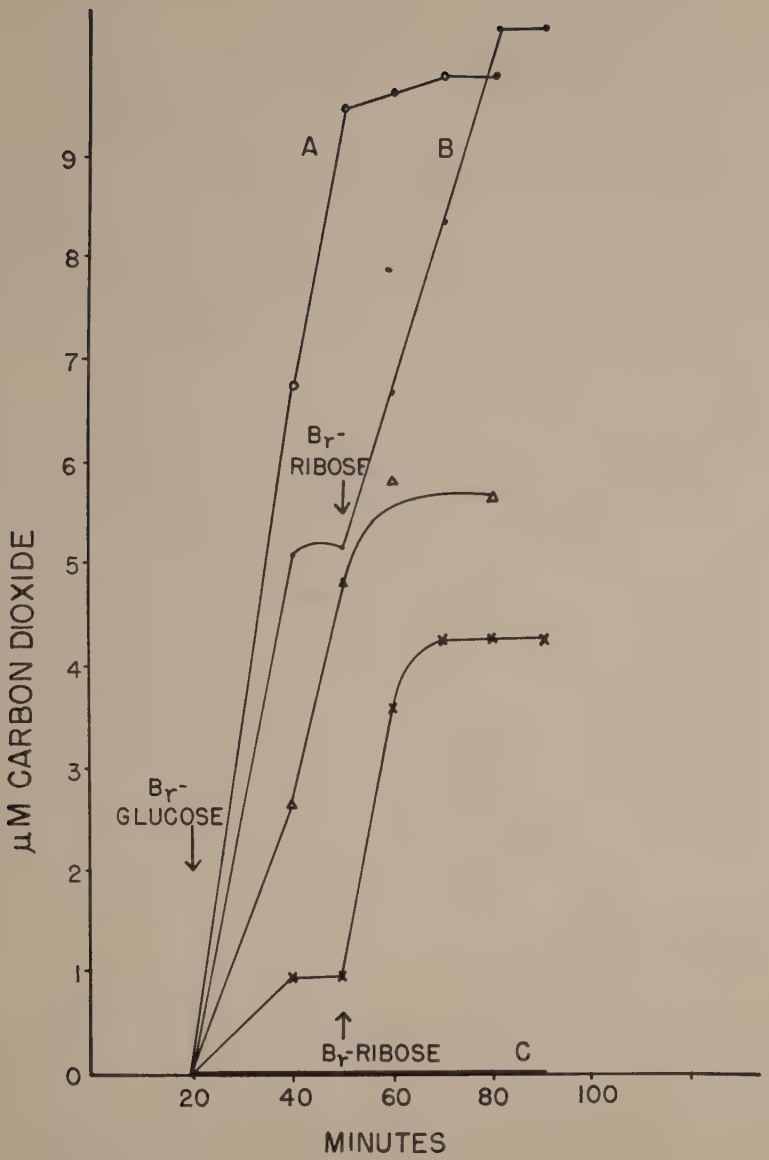


Fig. 8 Fermentation of various substrates by ribose-adapted and nonadapted *E. coli*, strain B_r

B_r — glucose = unadapted B_r grown on glucose.

B_r — ribose = B_r adapted by growth on glucose ribose mixtures.

The main vessel contained substrate in 2 ml of 0.01 *M* bicarbonate buffer.

Sidearm 1 contained B_r — glucose in 0.2 ml 0.01 *M* bicarbonate buffer.

Sidearm 2 contained B_r — ribose in 0.2 ml 0.01 *M* bicarbonate buffer.

Vessels were flushed with 5% CO_2 — 95% N_2 and incubated at 38°C.

Curve A = 800 μg glucose; curve B = 400 μg glucose + 400 μg ribose; curve C = 400 μg D-arabinose.

△ = 400 μg ribose + B_r-ribose.

× = Neutral fraction of dephosphorylated products of complete oxidation ≅ 251 μg ribose.

concentrations of alcohol, according to the method of Lew, Wolfrom, and Goepp ('46).

A method has recently been developed for the quantitative specific measurement of 2-ketogluconate by means of its reaction with o-phenylenediamine to form a quinoxaline which absorbs at 330 mμ (Cohen and Lanning, '50). In this way, 2-ketogluconate can readily be distinguished from 5-ketogluconate.

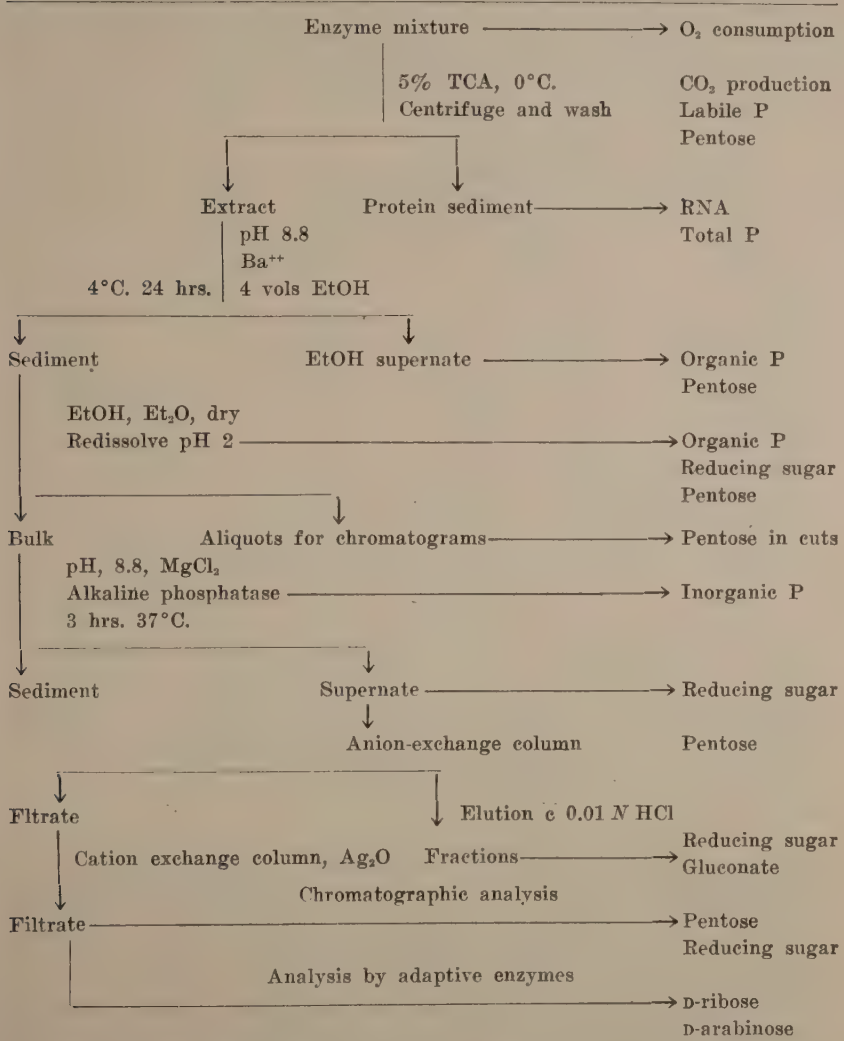
Pairs of adapted and nonadapted *E. coli* mutants have been developed for the specific manometric estimation of gluconate, D-ribose, and D-arabinose. The specificity of the gluconate-adapted coli is shown in figure 7. These bacteria fermented only glucose and gluconate.

In figure 8 are shown the specificity of the ribose mutant and the manometric technique used in an estimation. The unadapted organism fermented only glucose, even though ribose or D-arabinose were offered. On addition of the ribose-adapted organism from another sidearm after exhaustion of all glucose by the unadapted bacteria, a further fermentation occurred, specific for ribose. The ribose-adapted organism did not ferment D-arabinose. A different selected mutant of *E. coli* could be similarly prepared for the estimation of D-arabinose. By these methods, about 200-μg quantities of pentose could be measured within 3 to 5%.

Table 2 summarizes the fractionation and analysis of the reaction mixture. About 70-mg quantities of substrate were incubated with enzyme, TPN, dilute phosphate buffer, sodium cyanide, and phenazine for various lengths of time to give various levels of oxidation. The proteins were precipitated with trichloroacetic acid, which precluded identification of very labile phosphate compounds such as ribose-1-phosphate if they were present. The phosphates were almost completely precipitated as barium salts in 80% ethanol. Efforts to effect fractionations with barium and ethanol without treatment with trichloroacetic acid were variable and gave some interesting products which have not as yet been submitted to further analysis. The barium-alcohol precipitates were washed

TABLE 2

Fractionation and analysis of products of phosphogluconate degradation



with ethanol and ether to remove trichloroacetic acid, which affects the chromatograms, and then dried.

The dried barium salts were redissolved at pH 2 to 3 and aliquots were analyzed chromatographically. The rest of the barium salts were freed of barium and dephosphorylated by treatment with alkaline phosphatase at pH 8.8. The inorganic phosphate was precipitated as the magnesium salt

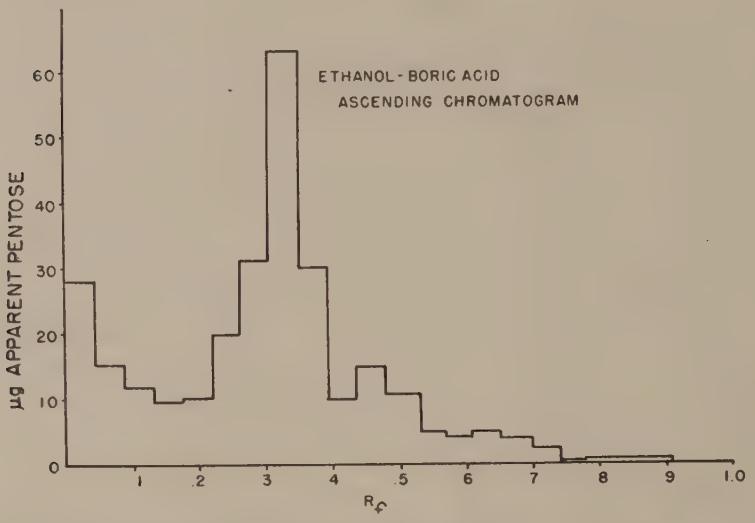


Fig. 9 The distribution of Bial-reactive phosphate in the intermediate oxidation product of phosphogluconate, in an ascending chromatogram developed in an ethanol-boric acid mixture. The paper was irrigated for about 25 cm and dried. One-centimeter strips were cut and eluted with 5 ml of water. Pentose was estimated in aliquots of the eluate.

and the supernatant fluid was passed through the anion-exchange column. The filtrate from the anion-exchange column was passed through the cation-exchange column, treated with silver oxide, and lyophilized. Aliquots were analyzed chromatographically and by adapted bacteria. The acid eluate from the anion-exchange column was collected in fractions which were analyzed chemically and by bacteria adapted to gluconate.

RESULTS

Chromatograms of the phosphates in ethanol-acetate have consistently revealed pentose phosphate with R_f 0.5 to 0.6, with the peak strongest at the ribose-5-phosphate position. Also, 6-phosphogluconic acid has been observed to decrease

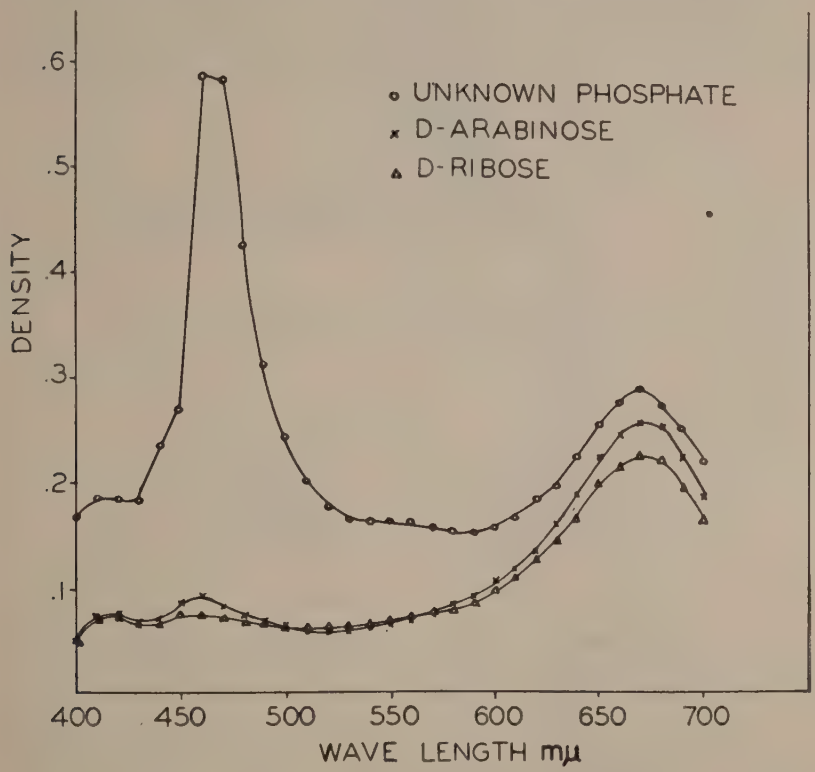


Fig. 10 Absorption spectra produced by the orcinol-$FeCl_3$ reaction with the unknown pentose phosphate compared with similar spectra for D-ribose and D-arabinose. This unknown Bial reactive phosphate was in the eluate from the chromatogram presented in figure 9 at R_f 0.32 to 0.36.

in amount and disappear as oxidation proceeded. When an intermediate oxidation product was chromatographed in ethanol-boric acid, which holds back ribose-5-phosphate, about one-half the apparent pentose was sharply revealed in a position somewhat more advanced than D-arabinose-5-phos-

phate (fig. 9). We say "apparent" pentose since analysis of the material at the peak produced a color in the Bial reaction which had a higher absorption maximum at 450 mμ than did any of the pentose phosphates (fig. 10, figs. 2 a–d). Reducing sugar and organic phosphate were also found in the same positions as "apparent" pentose. In the dephosphorylated filtrates of partially or completely oxidized materials, ribose and arabinose were revealed by the pentose

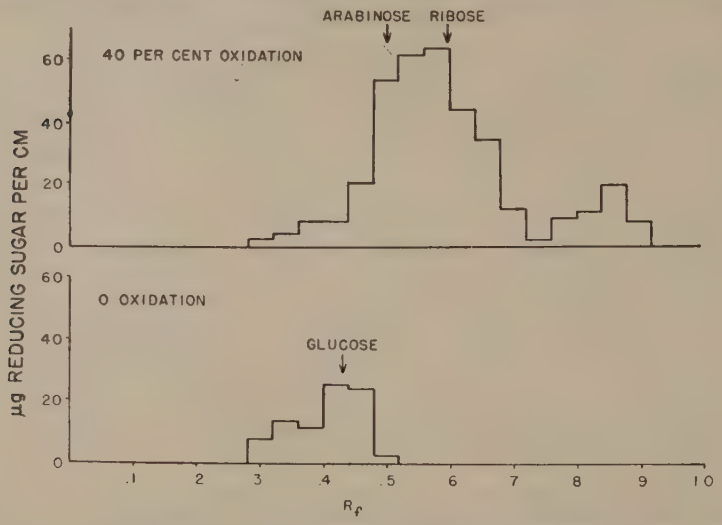


Fig. 11 The distribution of reducing sugars in paper chromatograms developed in *s*-collidine-H_2O. The spots contained dephosphorylated solutions of the phosphates isolated from reaction mixtures with phosphogluconate at 0 time and after 40 minutes reaction. The papers were irrigated for about 25 cm, cut and dried. One-centimeter strips were cut and eluted with 5 ml of water. Reducing sugar was estimated in aliquots of the eluates.

analyses of the chromatograms (fig. 11). The presence of these pentoses was also shown by analysis with adapted bacteria. Analyses with adapted bacteria revealed ribose to account for 25% of the total pentose (fig. 8), and D-arabinose for slightly less. This means that of the pentose present at the end point of 0.5 mole oxygen per mole phosphogluconate, about 50% was unknown pentose, about 25% was ribose, and the rest possibly arabinose.

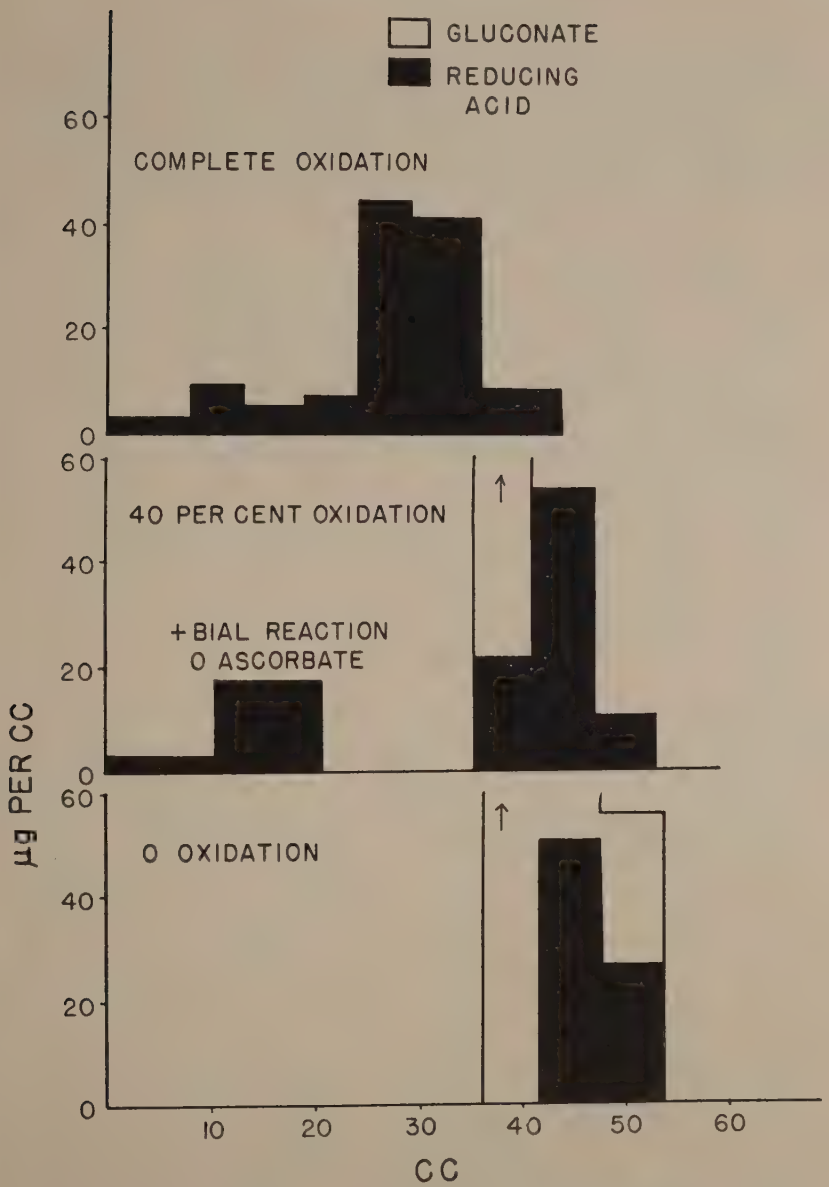


Fig. 12 The distribution of gluconate and reducing acid in eluates of anion-exchange columns. These columns had absorbed the acids from the dephosphorylated products of phosphogluconate reaction mixtures. The columns were eluted with 0.01 *N* HCl at flow rates of about 0.5 ml per minute.

In recent experiments using the new estimation of 2-ketogluconate, there was material reacting as 2-ketogluconate in the reaction mixtures. Later experiments have also sometimes shown spots on chromatograms which might be 2-ketogluconate. However, the question of whether a phosphate

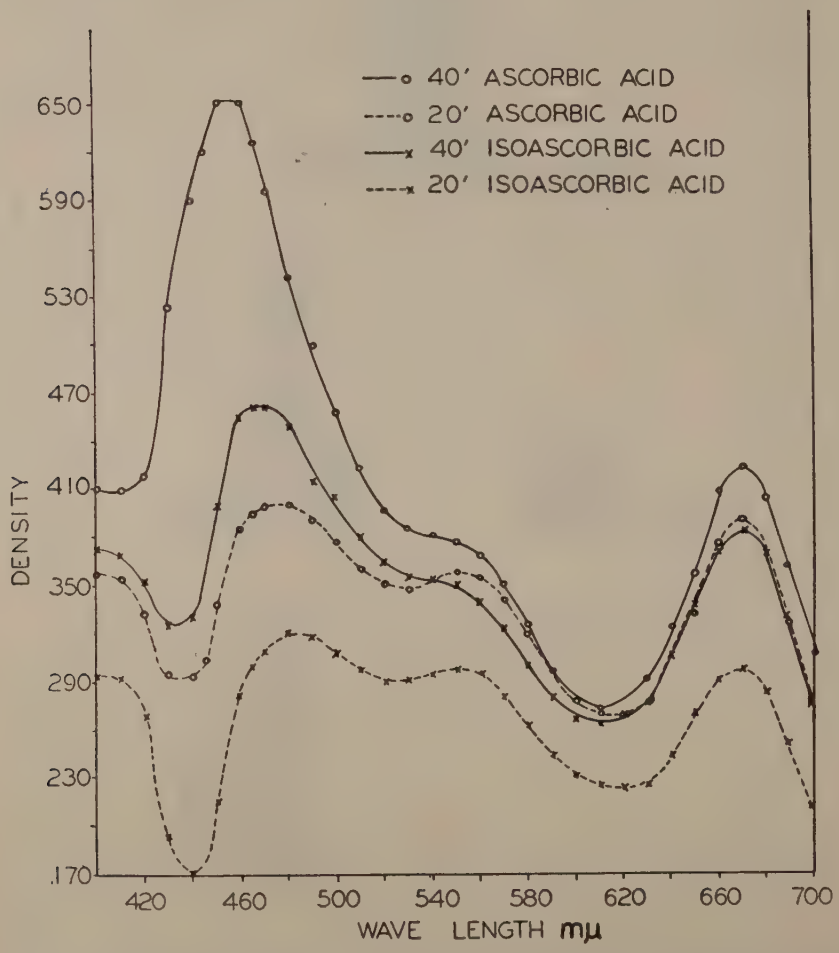


Fig. 13 Absorption spectra of the product of reaction of orcinol-$FeCl_3$ with ascorbic and isoascorbic acids. The Bial reaction mixture contained 29 μg isoascorbic acid per milliliter. The mixtures were heated at 100°C. for 20 or 40 minutes.

ester of 2-ketogluconate is an intermediate between the phosphogluconate and the pentose phosphate is still not settled unequivocally. Small amounts (1 to 3%) of 2-keto-6-phosphogluconate are formed in the bromine oxidation of glucose-6-phosphate and we have been unable to recover more than this amount of 2-ketogluconate at any stage of the reaction.

In figure 12 is shown an analysis of acids present in various oxidation products. Three points deserve comment: (1) gluconic acid disappeared as oxidation progressed; (2) only a small amount of reducing acid was present in these materials; and (3) a new, very weak acid appeared which gave pentose reactions and was adsorbed on the resin. This material was not an isoascorbate, the elution pattern of which was similar to gluconate, nor did it give the usual reactions of ascorbate. However, in the Bial reaction, and with o-phenylenediamine, it reacted in a manner similar to dehydroisoascorbate (fig. 13).

DISCUSSION

If all the phosphogluconate were converted to pentose, 0.5 mole of oxygen would be consumed and 1 mole of carbon dioxide would be given off; the RQ would be 2. The RQ would lie between 0 and 2 under certain conditions: (1) if the reaction did not go to completion and some 2-keto-6-phosphogluconate or other intermediate were present; or (2) if a side reaction were partially active which did not lead to decarboxylation. These relationships are outlined in figure 14. Either situation could account for our results.

Horecker ('50) has reported that with a purified enzyme preparation from yeast, 1 mole of carbon dioxide and 1 mole of pentose were produced per mole of phosphogluconate. Such a close correlation certainly did not occur with the acetic acid precipitated enzyme preparations described above. There sometimes was a correlation between carbon dioxide and apparent pentose production, but the RQ was always much less than 2.

Studies have been undertaken with enzymes from *E. coli* as well. It was possible to effect 95% disruption of the moist pellets of bacteria by grinding them by hand with certain fine grades of alumina in a mortar for two minutes. Phosphate buffer (0.01 *M*) at pH 7.0 eluted the bulk of the protein from this ground material. Potent hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and ribose-5-phosphate metabolizing systems have been found in these eluates, as well as the components of the Embden-Meyerhof cycle.

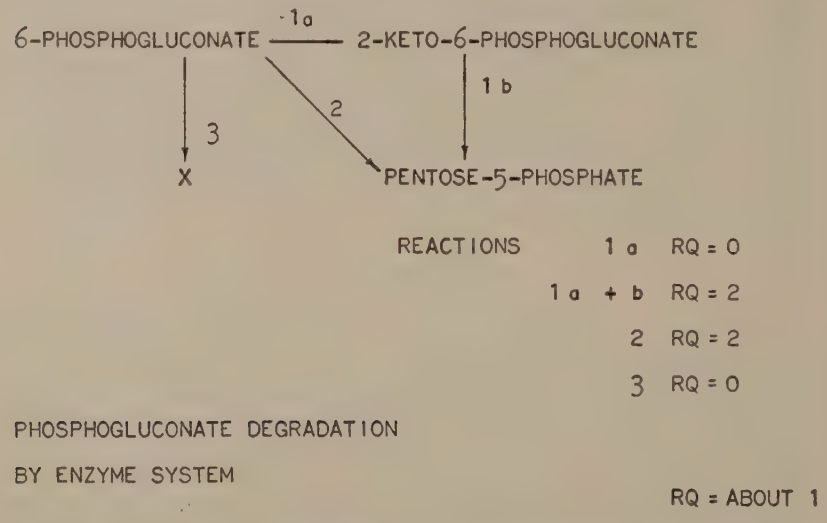


Fig. 14 Possible mechanisms of 6-phosphogluconate degradation.

Thus enzymes exist in yeast, apparently in *E. coli,* and probably in mammalian tissues for the transformation of glucose-6-phosphate to 6-phosphogluconate, which in turn can be degraded to pentose phosphate. Although the exact character of the primary pentose phosphate is not clear, ribose phosphate is thereby generated as well. It is suggested that the ribose phosphate is derived from the unknown primary pentose phosphate. Systems which are capable of transforming ribose phosphate to smaller fragments have been considered.

It is clear then that an oxidative pathway exists for the conversion of glucose-6-phosphate to carbon dioxide and ribose phosphate, thence to a two-carbon fragment and 1 mole of triose phosphate, which can then be metabolized in the usual fashion to carbon dioxide and other two-carbon fragments. This pathway exists side by side in many cells with the Embden-Meyerhof system for the anaerobic conversion of glucose-6-phosphate to fructose-1,6-diphosphate to 2 moles of triose phosphate. These relations are presented in figure 15.

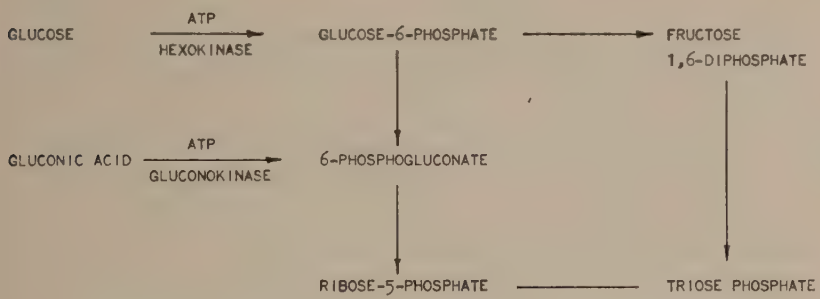


Fig. 15 Alternative paths of glucose-6-phosphate degradation.

Two important problems may then be posed: (1) what are the quantitative relations between the two alternative systems in normal cell growth, differentiation, tumor formation, virus infection, for example, and (2) how is the balance of the two systems controlled?

Recently, we have obtained evidence that in *E. coli* the oxidative pathway alone is capable of providing assimilable carbon and utilizable energy at a rate approaching that at which both the oxidative and the glycolytic systems are functioning. In adaptation to gluconate, a transphosphorylase, gluconokinase, is elaborated which in the presence of adenosinetriphosphate (ATP) and gluconate forms 6-phosphogluconate (Cohen and McNair Scott, '50b). In *E. coli* extracts, this substrate accumulates anaerobically, or aerobically in the absence of TPN. In growth on gluconate as the sole carbon source, that part of the Embden-Meyerhof system leading

to triose phosphate formation is by-passed (see fig. 15). In growth on glucose as the sole carbon source, both systems may be active, since hexokinase and ATP provide glucose-6-phosphate.

The 6-phosphogluconate dehydrogenases have similar activities in both adapted and nonadapted cells. Since the mass duplication time of *E. coli* on gluconate is 57 minutes, as contrasted to 49 minutes in growth on glucose, we have concluded that in growth on glucose, the oxidative pathway in *E. coli* is potentially as important, if not more important, than the Meyerhof system. This is not unexpected in an organism that synthesizes about 15% of its weight as ribonucleic acid.

Nevertheless, it is clear that a study of cell-free enzymes can do little more than suggest the relative importance of these systems in actually functioning cells. The importance of the problem is so clear that without a doubt dozens of ingenious experimental solutions will be devised in the next period. One possible approach may involve the use of glucose labeled with isotopic carbon in positions 1, 2, or 3, since such labels can serve to indicate the origin of carbon dioxide and a two-carbon fragment derived from the oxidative pathway, in contrast to that derived from the triose phosphated generated in anaerobic glycolysis.

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B-VITAMINS AND THE BIOSYNTHESES OF PURINES AND PYRIMIDINES

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ONE FIGURE

INTRODUCTION

Competitive inhibition of enzymatic reactions was probably first observed as early as 1892 (Tammann, 1892; Armstrong, '04) when products of certain enzymatic reactions were reported to exert an inhibitory action on the specific enzymes which catalyzed these reactions. The inhibitions obtained in such instances apparently are not the result of mass action effects in reversing the reactions but are the result of the formation of complexes between the enzymes and the products of the reaction. Such complexes are presumably formed in much the same manner as those between enzyme and substrate as first indicated by Michaelis and Menten ('13). That compounds structurally related to the substrate may inhibit the action of a specific enzyme on that substrate by forming a complex with the enzyme was not generally realized until malonic acid and compounds structurally related to succinic acid were reported by Quastel and Woolridge ('28) to inhibit the dehydrogenation of succinic acid to fumaric acid by succinic dehydrogenase. In 1940 Woods and Fildes ('40) reported that p-aminobenzoic acid, a compound synthesized in 1863 (Fischer, 1863), competitively prevented for certain bacteria the toxicity of sulfanilamide, a compound synthesized in 1908 (Gelmo, '08) but not found to be a chemotherapeutic agent until 1935 as a result of studies on the mode of action of prontosil (Domagk, '35; Tréfouel et al., '35).

Aside from the discovery that p-aminobenzoic acid is a catalyst in certain biochemical processes, this report led to the realization that compounds structurally similar to metabolites have broad application in chemotherapy, pharmacology, and related fields and prompted a widespread search for inhibitory analogues of metabolites.

The development of testing techniques for the study of biochemistry with competitive inhibitory analogues of metabolites was undertaken in our laboratories about 5 years ago (Shive and Macow, '46). Although many research workers had previously indicated the possibility of using competitive inhibitors of metabolites in the elucidation of biochemical mechanisms, relatively few studies using such compounds had actually been made. A fundamental approach to the problem has led to the development of techniques which have broad application and which in particular have been used to demonstrate the involvement of certain B-vitamins in the biosynthesis of purines and pyrimidines.

Theoretical considerations. The mechanism of action of an enzyme is such that a complex is formed with the substrate. This complex decomposes to form the product of the reaction. If a compound analogous in structure to the substrate can also form a similar complex with the enzyme, the enzyme can be prevented from acting upon the substrate by such an analogue. The interaction of the enzyme with either the substrate (S) or the analogue (I) can be represented by the following equations analogous to the original equations of Michaelis and Menten ('13):

$$\begin{aligned} E + S &\rightleftharpoons ES \rightarrow P + E \\ E + I &\rightleftharpoons EI \end{aligned}$$

where E represents the enzyme, ES and EI represent the enzyme-substrate complex and enzyme-inhibitor (analogue) complex, respectively, and P represents the product. By the Law of Mass Action,

$$\frac{[E][S]}{[ES]} = K_s \qquad (1)$$

where K_S is the dissociation constant of the enzyme-substrate complex, and

$$\frac{[E][I]}{[EI]} = K_I \qquad (2)$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. By dividing equation 2 by equation 1,

$$\frac{[I]}{[S]} = \frac{K_I [EI]}{K_s [ES]}. \qquad (3)$$

The total enzyme concentration, $[E_t]$, can be represented as the sum of the concentrations of free enzyme, E, and the combined forms, ES and EI; hence,

$$[E_t] = [E] + [ES] + [EI]. \qquad (4)$$

Solving equation 4 for [EI], and substituting the value in equation 3, one obtains the following equation:

$$\frac{[I]}{[S]} = \frac{K_I \Big\{ [E_t] - [ES] - [E] \Big\}}{K_s \qquad [ES]}. \qquad (5)$$

In order to inhibit the enzymatic process to any appreciable degree, the concentration of free enzyme must be reduced to a relatively low value compared with the concentration of enzyme-inhibitor complex. Also, under conditions such that the concentrations of inhibitor and substrate are being increased, $[E] \rightarrow 0$ and is negligible in determining the value of [EI] in equation 4. In the application of these equations to the study of biological systems (Shive and Macow, '46), the rate of the biological process is a function of the rate of the inhibited reaction which is the limiting reaction of the biological process. If a defined inhibition of some observable effect, e.g., growth of the biological system, is attained after a defined experimental period, a certain defined initial rate of the inhibited reaction is required for the final defined response.

Since the rate of the inhibited reaction is proportional to the concentration of the enzyme-substrate complex, it is necessary to reduce the concentration of the enzyme-substrate complex to a defined value, C_{ES}, corresponding to that neces-

sary for the defined response of the biological system. This can be accomplished by varying the concentration of inhibitor. The total enzyme concentration [E_t] can be controlled at a constant value or can be expected to be relatively constant in most biological systems in which concentrations of inhibitor and substrate are varied. During growth, mother and daughter cells would normally have essentially the same enzyme concentration. Consequently, the concentration of the enzyme-inhibitor complex will be essentially a defined value, C_{EI}, if the concentration of free enzyme is considered to be negligible. The following equation is obtained by substitution of these values in equation 3:

$$\frac{[I]}{[S]} = \frac{K_I \ C_{EI}}{K_S \ C_{ES}} = K \qquad (6)$$

where K represents the ratio of the concentration of inhibitor to substrate at which the response of the biological system is reduced to a defined value. For experimental conditions in which the concentrations of inhibitor and substrate are regulated externally, the internal ratio of the concentrations would be expected to be a function of the external ratio. This ratio necessary for the defined response has been termed the "inhibition index."

Inhibition analysis. A number of techniques have been developed for the study of biochemistry with competitive inhibitors of substrates (metabolites) (Shive, '49). Usually these involve the effects of substances other than the substrate (metabolite) on inhibited biological systems. Such a biological system is not limited to any specific phylum and does not depend upon genetic deficiencies in the experimental organism. The method is applicable to the study of any enzymatic process in a living organism as well as to systems *in vitro*.

The types of substances other than the metabolite itself which may affect a biological system which is competitively inhibited by an analogue of a metabolite include: (1) precursors of the metabolite, (2) the product of the inhibited enzyme, (3) substances which exert a "sparing effect" on

the product, (4) compounds which increase the effective enzyme concentration, and (5) substances which increase or decrease the rate of destruction of inhibitor or the biological compounds involved in the system.

Since a limiting precursor increases the concentration of the substrate in a biological system, the final concentration of the substrate is the sum of the contribution by the organism through synthesis and the contribution from exogenous substrate. If the concentration of exogenous substrate is very low, a limiting precursor supplied to the biological system may exert a very marked effect such that several times the amount of inhibitor normally necessary for a defined inhibition will be required. However, in the presence of high concentrations of exogenous substrate such that synthesis by the biological system even in the presence of the limiting precursor contributes little to the effective substrate concentration, the limiting precursor will no longer exert an appreciable effect on the inhibited biological system. Hence, the effect of limiting precursors and other substances which alter the concentration of substrate synthesized by the biological system will be "diluted out" by high concentrations of the substrate (metabolite).

An example of such an effect (Beerstecher and Shive, '46) is offered by the action of tryptophane on the inhibition of growth of *Escherichia coli* by β-hydroxyphenylalanine, a competitive inhibitor of phenylalanine. Complete inhibition of growth of *E. coli* occurs at an inhibition index of approximately 1000. While growth of the organism in an inorganic salts-glucose medium was almost completely inhibited by β-hydroxyphenylalanine at a concentration of 30 μg per cubic centimeter, approximately 1000 μg per cubic centimeter of the inhibitor was required for the same degree of inhibition if the medium was supplemented with 20 μg per cubic centimeter of DL-tryptophane. If tryptophane functions as a limiting precursor, the increase in synthesis of phenylalanine by the organism would be equivalent to an exogenous concentration of 1 μg per cubic centimeter. If supplementary

phenylalanine in concentrations of 1 to 3 μg per cubic centimeter were added to the medium, the synthesis from tryptophane would not be expected to exert an appreciable effect. Such is the case since tryptophane (20 μg per cubic centimeter) does not affect the inhibition of growth of the organism in the presence of phenylalanine at concentrations of greater than 1 μg per cubic centimeter. Consequently, supplementary tryptophane allows the organism to maintain a higher concentration of phenylalanine in the cell. Substances which exert a precursor effect include limiting precursors, limiting coenzymes required for synthesis of the substrate (metabolite), or substances which prevent the destruction of the metabolite in ways which are not essential for the biological response.

If the product, or its equivalent of an inhibited enzymatic reaction can be and is supplied exogenously to the biological system, the inhibited enzymatic reaction will no longer be essential for the system. Consequently, the response of the system will no longer be inhibited by the analogue of the substrate unless still another enzyme utilizing the substrate is also inhibited by the analogue. Thus, it is possible that a series of different enzymes, such as E_1, E_2, E_3, which convert the substrate to several products, as P_1, P_2, P_3, may also form enzyme-inhibitor complexes with the analogue. If such is the case, one particular enzyme system, E_1 for example, will become the first limiting system of the biological process. The specific equilibrium constants and related factors for this enzyme system result in a specific inhibition index, K_1, which is a property of this particular enzyme system and is the lowest of the inhibition indices of all the enzyme systems for which the metabolite and its analogue compete. If the product, P_1, can be and is supplied to the biological system from an external source, the inhibition index determined in the presence of this product would be related to the next limiting reaction in which another product, P_2 for example, is formed from the substrate. This inhibition index, K_2, would be related directly to this enzyme

system, E_2. Consequently, a series of increasing inhibition indices are obtained as the products of the inhibited enzyme systems are supplied exogenously. Products of enzyme systems with higher inhibition indices would not be expected to exert any effect in the absence of those of enzyme systems with lower inhibition indices. A definite order exists in which these products must be added in order to affect the response of the inhibited biological system.

Either β-alanine or pantothenic acid at concentrations slightly higher than the lowest concentrations giving any effect completely prevents for *E. coli* the toxicity of cysteic acid, but aspartic acid prevents the toxicity competitively (Ravel and Shive, '46). Hence, it appears that cysteic acid competes with aspartic acid for the enzyme which decarboxylates the latter compound to β-alanine. This inhibition has now been confirmed with a cell-free enzyme system derived from *E. coli* (Rowen and Shive, '50). No other function of aspartic acid appears to be prevented by cysteic acid in that organism. On the other hand, β-alanine or pantothenic acid affects the inhibition of growth of *E. coli* resulting from the action of β-hydroxyaspartic acid, another competitive inhibitory analogue of aspartic acid, but does not completely prevent the toxicity of the analogue (Shive and Macow, '46). The inhibition index determined over a range of concentrations of aspartic acid is increased from approximately 3 to 20 by exogenous β-alanine or pantothenic acid. Consequently, it appears that in *E. coli* at least one enzymatic reaction of aspartic acid other than decarboxylation to β-alanine is inhibited by β-hydroxyaspartic acid.

If a substance acts in such a manner that the defined response of the biological system can be attained with less than the normal requirement of the product of the inhibited enzymatic reaction, it is apparent that a further decrease in the rate of the enzymatic reaction is necessary in order to maintain the defined inhibition of the biological system (Harding and Shive, '48). The rate of this reaction can be decreased only by decreasing the concentration of the enzyme-substrate

complex. From equation 5, it is apparent that a corresponding increase in the ratio of inhibitor to substrate concentrations, the inhibition index, is necessary in order to maintain the defined inhibition. Substances which exert such "sparing effects" are well known and include secondary products which are derived directly from the primary product and products of enzymatic reactions utilizing the coenzyme derived from an inhibited enzyme system which converts a vitamin to its coenzyme form. In each of the above, these secondary products would exert their effects in a definite order depending upon the sequence in which each becomes the limiting product necessary for the biological system.

The tricarboxylic acids, citric, *cis*-aconitic, isocitric acids, and α-ketoglutaric acid exert such a sparing effect on pantothenic acid in *E. coli*. The inhibition index obtained with cysteic acid inhibition of the decarboxylation of aspartic acid is increased from approximately 300 to 3,000 by supplementing the medium with any of this group of acids, but oxalacetic acid and pyruvic acid or acetic acid exert only a slight effect which is not comparable with the tricarboxylic acids or α-ketoglutaric acid. Consequently, it appears that decreased production of β-alanine results in a decrease in the coenzyme form of pantothenic acid (coenzyme A). The first essential enzyme system affected by this decrease is the system which converts oxalacetic and acetic acids to citric acid. If the products of this enzyme reaction are supplied to the organism, the enzyme is no longer essential for growth. Since the pantothenic acid requirement for all other essential enzyme systems is markedly lower, an increase in the ratio of cysteic acid to aspartic acid to decrease the rate of β-alanine formation is essential for the same degree of inhibition. Cells of *E. coli* which are inhibited by cysteic acid have been found to contain decreased amounts of the complete enzyme system which catalyzes the formation of citric acid from oxalacetic and acetic acids (Rowen and Shive, '50).

From equation 5, it is apparent that an increase in the total enzyme concentration would necessitate an increase in

the ratio of inhibitor to substrate concentrations if the concentration of enzyme-substrate complex (and the biological response) is to be maintained at a defined value. Substances which produce such a change in the effective enzyme concentration include the coenzyme (if limiting) of the inhibited enzyme or precursors of this limiting coenzyme as well as a limiting second substrate which interacts with the inhibited substrate.

Substances which change the normal rate of destruction of the inhibitor or other substances involved in the inhibited enzyme system affect the enzyme system in several ways, but these types of substances are less frequently encountered.

While reversing agents of types 2, 3, and 4 cannot as yet be distinguished in isolated cases, it is possible to differentiate to some extent between such effects if all are obtained in the same system. Reversing agents of types 3 and 4 exert no effect after the addition of the immediate product, type 2, even though an additional enzyme system may still be involved. Agents of types 3 and 4 are synergistic in preventing the toxicity of the competitive inhibitor. Data which involve the equilibrium constants of the enzyme system can be used to differentiate agents of type 2 from those of types 3 and 4 since the same enzyme is still involved in types 3 and 4, but different enzymes are involved in case of reversing agents of type 2.

BIOCHEMICAL FUNCTIONS OF P-AMINOBENZOIC ACID AND FOLIC ACID IN RELATIONSHIP TO THE BIOSYNTHESIS OF PURINES AND PYRIMIDINES

Various substances affecting the inhibitory action of sulfonamides have been known for some time (Loomis et al., '41; Kohn and Harris, '41). Methionine (Kohn and Harris, '41a, b; Bliss and Long, '41; Harris and Kohn, '41) over a limited range of concentrations is known to prevent the toxicity of sulfonamides for *E. coli,* and the effectiveness of methionine is enhanced by purines. Adenine and hypoxanthine were reported to be as effective as p-aminobenzoic acid in preventing

the chemotherapeutic effect of sulfanilamide against infections of *Streptococcus hemolyticus* in mice (Martin and Fisher, '42). For the lactic acid bacteria (Snell and Mitchell, '43) and *Eremothecium ashbyii* (Schopfer and Guilloud, '46), purines under specific conditions prevent the toxicity of sulfonamides. While these effects have been cited as evidence against the competitive analogue-metabolite theory of sulfonamide action, adequate explanations for such actions are offered by the theory of "inhibition analysis."

In such a study of the inhibition of growth of *E. coli* by sulfanilamide (Shive and Roberts, '46; Winkler and de Haan, '48; Shive, '49), the ratio of sulfanilamide to p-aminobenzoic acid just necessary to prevent growth of the organism was approximately 3000 in an inorganic salts-glucose medium. However, this ratio increased to approximately 10,000 when the medium was supplemented with adequate amounts of methionine. The amount of methionine required to exert this effect was approximately equivalent to that necessary for growth of mutant strains of the organism which require methionine for growth. Concentrations of methionine in excess of these amounts did not further affect the inhibition. Consequently, it appears that sulfanilamide prevents the functioning of p-aminobenzoic acid in an enzyme system necessary for the biosynthesis of methionine. This presumably occurs by inhibition of coenzyme formation since p-aminobenzoic acid is a catalytic factor. Homocysteine which is usually considered to be the immediate precursor of methionine does not replace methionine under these conditions (Winkler and de Haan, '48; Shive, '49), but this precursor does have an effect on the toxicity of certain methionine analogues and on certain mutant strains of *E. coli*. Thus, it appears that p-aminobenzoic acid functions in the methylation reaction in the conversion of homocysteine to methionine as indicated in figure 1.

The purine bases or their derivatives (e.g., ribosides) do not exert an effect on the inhibition of *E. coli* by sulfanilamide in the inorganic salts-glucose medium; however, if methio-

nine is present in the medium in adequate amounts, the inhibition index increases from approximately 10,000 to 30,000 when the purine bases or their derivatives are added to the medium in amounts approximating that necessary for growth of mutant strains of the organism requiring purines for growth. Higher concentrations of the purines or their derivatives have no additional influence on the inhibitory action of sulfanilamide. Xanthine is the most effective purine base, but both guanine and hypoxanthine have some activity

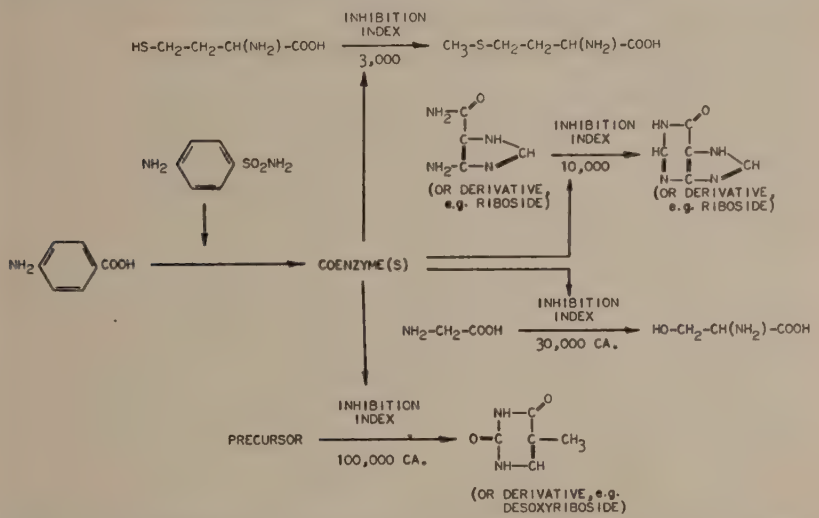

Fig. 1 Interrelationships of p-aminobenzoic acid as determined by inhibition analysis with sulfanilamide.

in preventing the toxicity of sulfanilamide for *E. coli*. Adenine is usually toxic for the organism under these conditions, but adenosine and other purine ribosides are very active in preventing the toxicity of the inhibitor. Thus, adenine appears to have an inhibitory action which involves another closely related system, but the riboside of this purine, not having this inhibitory action, parallels other purines and their derivatives in their ability to prevent the toxicity of sulfanilamide under these conditions. The purine bases or their

derivatives appear to be interchangeable for *E. coli* in supplying the product of a system inhibited by sulfanilamide.

Since the biosynthesis of purines appears to be prevented by sulfanilamide, the possibility of the accumulation of a precursor of purines in the medium under conditions such that purine synthesis is the limiting factor for growth was considered. An unidentified amine was previously reported to accumulate in the growth medium of *E. coli* and other organisms in the presence of sulfonamides. This amine was isolated (Stetten and Fox, '45) and identified as 5(4)-amino-4(5)-imidazolecarboxamide (Shive et al., '47). From these results, it appears that in *E. coli* 5(4)-amino-4(5)-imidazolecarboxamide is either a precursor of purines or is formed from a precursor of purines. As the conversion of this amine to purines requires a condensation with a formate group, it appears that p-aminobenzoic acid functions in such a condensation reaction as indicated in figure 1.

5 (4) - Amino - 4 (5) - imidazolecarboxamide stimulates the growth of *Lactobacillus arabinosus* in a manner similar to purines and disappears from the medium during growth of the organism. However, since the amine is not utilized by many organisms, it seems that it may not itself be the immediate precursor of purines but that a conjugated derivative, e.g., riboside, may be the normal intermediate which accumulates and is converted by *E. coli* to the unconjugated amine.

Many organisms, particularly the lactic acid bacteria, do not produce the aminoimidazolecarboxamide in the presence of inhibitory analogues of folic acid or p-aminobenzoic acid. Most of these organisms are capable of utilizing folic acid. However, the aminoimidazolecarboxamide increases slightly the sulfanilamide inhibition index for *L. arabinosus* in the absence of purines, but purines prevent the toxicity at even higher ratios of inhibitor to p-aminobenzoic acid. The possibility exists that in some organisms such as these, the introduction of the single carbon unit in the imidazole ring becomes the first limiting reaction of purine synthesis, while

in others, the formation of the pyrimidine ring is the first limiting reaction resulting in an accumulation of an imidazole derivative.

In a study of the effect of amino acids on the production of the aminoimidazolecarboxamide (Ravel et al., '48), glycine was found to increase the amount of the amine formed. The amount of the amine formed is directly related to the concentration of glycine present in the medium, and this conversion represents a major pathway of glycine metabolism in *E. coli* under these conditions. Glutamic acid enhances the production of the amine from glycine but does not replace glycine. The only amino acid which replaces glycine under these conditions is threonine, and it is only about 10% as active as glycine. Since the formation of threonine from glycine has been observed in yeast (Rossi and Cennamo, '45), threonine apparently is converted to glycine and a two-carbon fragment by the organism under these conditions.

Serine which is known to be interchangeable with glycine for certain mutant strains of *E. coli* (Roepke et al., '44) did not affect the production of the amine. These results suggest that the interconversion of glycine and serine is inhibited by sulfonamides and that a coenzyme derived from p-aminobenzoic acid is involved in this reaction.

Later, it was shown that serine under certain conditions affected the inhibition of growth of *E. coli* by sulfanilamide, but this effect was observed with the organism only in the presence of supplements of both methionine and purines (Winkler and de Haan, '48). The inhibition index is increased from approximately 30,000 to 100,000. These data furnished further evidence that a coenzyme derived from p-aminobenzoic acid is involved in the conversion of glycine to serine.

Thymine in the presence of serine, purines, and methionine exerts an additional effect on the inhibition by increasing the ratio to approximately 200,000. Folic acid is somewhat interchangeable with thymine (Winkler and deHaan, '48). From these results, it is apparent that p-aminobenzoic acid

is involved in the biosynthesis of methionine, purines, serine, and thymine.

It also appears from these results that p-aminobenzoic acid is involved in a series of reactions involving the introduction of a single-carbon unit into methionine, purines, and serine. A close relationship of these single-carbon units is suggested by these results and has been further indicated by isotopic work. Thus, isotopically labeled formate is incorporated into the 2 and 8 positions of uric acid (Buchanan and Sonne, '46; Sonne et al., '48), into serine as the β-carbon atom (Sakami, '48), and into the methyl groups of methionine and choline (Sakami, '50; Welch and Sakami, '50).

The interrelationship of folic acid and p-aminobenzoic acid in *E. coli* is somewhat complicated. Folic acid exerts a noncompetitive reversal of sulfonamide toxicity only in the presence of methionine, purines, and serine. Hence, folic acid cannot account for all the biochemical functions of p-aminobenzoic acid in *E. coli.* The question of whether there is only one or more than one coenzyme derived from p-aminobenzoic acid remains to be determined. It is possible that folic acid is not the normal intermediate in coenzyme formation from p-aminobenzoic acid and that the ability of this organism to convert folic acid to the normal coenzyme is limited to such an extent that the organism cannot synthesize from folic acid the larger concentrations necessary for biosynthesis of methionine, purines, and serine. However, the small amount of coenzyme necessary for the enzyme system involved in the biosynthesis of thymine could possibly be derived from folic acid rather than p-aminobenzoic acid and thereby account for the effect of folic acid. As expected, other organisms have been found to utilize folic acid more effectively. If, on the other hand, it is assumed that several coenzymes are derived from p-aminobenzoic acid, it must also be assumed that folic acid is readily convertible to these coenzymes since certain streptococci utilize folic acid just as effectively as p-aminobenzoic acid. Also, these organisms must have for the con-

version of folic acid to these coenzymes pathways which are not inhibited by sulfonamides, since sulfonamides inhibit the utilization of p-aminobenzoic acid but not that of folic acid.

Some clarification of the interrelationship of these two vitamins has been obtained by a study of the function of folic acid in organisms requiring the vitamin for growth. Both thymine and purines are required in the absence of exogenous folic acid for both *Streptococcus faecalis* R and *L. casei* (Snell and Mitchell, '41; Stokstad, '41; Stokes, '44). Similarly, the inhibition index obtained with x-methylfolic acid preventing the functioning of folic acid in *L. casei* is increased from 30 to 100 by a purine supplement and to 1000 by both a purine and thymine supplement (Rogers and Shive, '48). Similar results are obtained with *S. faecalis* R except that mixtures of purines and thymine completely prevent the toxicity of the inhibitor (Stokstad et al., '48). Both serine and glycine become essential for growth of *S. faecalis* R under these conditions, whereas in the presence of exogenous folic acid this organism requires only serine or glycine (Holland and Meinke, '49). Hence, the biochemical reactions catalyzed by the two vitamins appear to be identical, and a single coenzyme derived from either vitamin functioning as the carrier of the single-carbon unit appears to be the most likely explanation.

In a search for some functional derivative of folic acid which might serve as a carrier of such a unit, pteroylhistidine was synthesized and found to be without any unusual activity. However, the announcement of the structure of rhizopterin as formylpteroic acid (Wolf et al., '48) led to the preparation of formylfolic acid as a possible functional form of folic acid (Gordon et al., '48). This formyl derivative is approximately 30 times as effective as folic acid in preventing the toxicity of x-methylfolic acid for *S. faecalis* R. These results further indicate that the role of folic acid is that of a carrier of the single-carbon unit.

THE ANTIPERNICIOUS-ANEMIA PRINCIPLE OF LIVER AND ITS RELATIONSHIP TO THE BIOSYNTHESIS OF PURINES AND PYRIMIDINES

Since the original work of Minot and Murphy ('26), liver has been known to contain a principle(s) effective in alleviating pernicious anemia. More recently folic acid has been found to be effective in relieving the anemia but not the neurological symptoms of the disease. Also some liver preparations, even though impure, were considerably more active than pure folic acid. Obviously there was in these liver extracts some factor(s) functionally related to folic acid. This situation offered an ideal opportunity for demonstrating how "inhibition analysis" could be used in developing assays for unknown principles biochemically related to a known metabolite. By using a large number of inhibitors related to purines, pyrimidines, folic acid, and p-aminobenzoic acid, more than 20 assays for principles which were not identical with any known nutritional factor were developed.

Thymidine. One such assay (Shive et al., '48) was based on the ability of a principle in liver extract to increase the ratio of x-methylfolic acid to folic acid from 3000 to 30,000 for complete inhibition of growth of *Leuconostoc mesenteroides* 8293. As little as 1 μg of liver extract (20 units per cubic centimeter) gave a half-maximal response under assay conditions. These results indicated that folic acid functioned in the biosynthesis of the active principle; consequently, the factor appeared to have some possibility of being the antipernicious-anemia principle, and isolation of the factor was begun.

During the isolation of this factor, it was observed that inhibition of growth of *Lactobacillus arabinosus* by 2,4-diamino-6,7-diphenylpteridine was also prevented by liver extracts or concentrates of the factor which reversed the toxicity of methylfolic acid for *L. mesenteroides* 8293 (Shive, '49). It was soon obvious that the same factor was effective in both assays. Although the assay using the pteridine inhibitor was less sensitive (0.1–0.2 μg per cubic centimeter of pure factor giving a half-maximal response), the slope of the dose-

response curve for this compound was more suitable for a highly specific quantitative assay.

Another assay for this same factor depended upon the ability of the principle to prevent the toxicity of sulfonamides (Shive, '49). Thus three assays were independently developed for this factor, which was subsequently isolated from liver digests and identified as thymidine, the desoxyriboside of thymine (Shive et al., '48).

The assay using the pteridine inhibitor is highly specific and is not affected by folic acid, vitamin B_{12}, any of the other desoxyribosides, or thymine.

Antipernicious-anemia principles. Another assay was developed on the basis of the ability of a factor in liver extracts to prevent toxicity of sulfanilamide for *E. coli* under conditions such that the biosynthesis of methionine limited the growth response of the organism (Shive, '49). Employing this assay the isolation (Shive, '49) of a red crystalline compound was accomplished shortly after the announcement of the isolation of vitamin B_{12} (Rickes et al., '48; Smith and Parker, '48). As vitamin B_{12} was not available for comparison, the name erythrotin was given the red compound which was found to be an effective erythrocyte maturation factor in the treatment of pernicious anemia. A half-maximal response of *E. coli* under the testing conditions was obtained with 0.00001 μg of crystalline erythrotin per cubic centimeter. The organism also responds to methionine under these conditions, but erythrotin is about 300,000 times as active as methionine; consequently, there is little interference in the assay of materials which contain any appreciable amount of erythrotin. Erythrotin increases the sulfonamide inhibition index approximately threefold whether the limiting reaction is the biosynthesis of methionine, purines, serine, or thymine. Thus, erythrotin (or the vitamin B_{12} group) functions either in the conversion of p-aminobenzoic acid to its active coenzyme form or, independently, in the series of biochemical transformations involving a single-carbon unit.

Under conditions of limiting purine synthesis, erythrotin and purines are synergistic in reversing toxicity of the sulfonamide for *E. coli*. In the presence of suboptimal concentrations of purines, as little as one part of erythrotin in 1,000,000,000,000 parts of medium gives an excellent growth response of the organism (Shive, '50b). On incubation for 18 hours, rather than for 12–13 hours, either erythrotin or purines at higher levels give a maximal response. An assay technique developed along these lines allows detection of as little as a fraction of one-millionth of 1 μg of erythrotin per cubic centimeter. This test has been useful to R. E. Eakin in our laboratories in the study of apoerythein (Ternberg and Eakin, '49), a protein which combines with erythrotin to form a red protein complex, erythein, which does not diffuse into the cells of certain microorganisms. This protein is detected by its ability to prevent the utilization of vitamin B_{12} by microorganisms normally responding to the vitamin. By using such a sensitive assay, apoerythein can be detected in a few micrograms of washed tissue from the gastrointestinal tracts of vertebrates.

The folinic acid group. In reversing the toxicity of x-methylfolic acid for *L. casei,* liver extracts were observed to be at least 15 times as effective as the expected activity from the amount of folic acid, as determined by microbiological assay, in the extracts (Bond et al., '49). Using the reversal of the toxicity of x-methylfolic acid for *L. casei* as a testing method, an assay was developed with the usual medium for lactic acid bacteria but supplementing the medium with x-methylfolic acid (1 μg per cubic centimeter), folic acid (0.001 μg per cubic centimeter), and thymine. The active principle was isolated in essentially pure form and found to give a half-maximal response at 0.0001 to 0.0002 μg per 10 cc. While this work was in progress liver extracts were reported (Sauberlich and Baumann, '48) to contain a factor essential for the growth of *Leuconostoc citrovorum* 8081. It was proposed that this factor might be identical with vitamin B_{12}. However, crystalline erythrotin did not replace the liver ex-

tract, and fractions of liver extract from which the thymidine had been removed were relatively inactive (Bardos et al., '49). This suggested the possibility that the high activity of liver extracts might be the result of a synergistic action of two components, one of which was thymidine. In the presence of thymidine (0.1 μg per cubic centimeter of medium) folinic acid was observed to be highly active in promoting the growth of *L. citrovorum* 8081, with a half-maximal response resulting from the addition of 0.00001 to 0.000015 μg per cubic centimeter. However, in the absence of thymidine, a slightly different-shaped growth response curve was obtained with a half-maximal response occurring with a supplement of 0.0005 to 0.00075 μg per cubic centimeter. This synergistic effect of thymidine on the response to folinic acid indicates the possibility that thymidine may be involved in the conversion of folinic acid to its normal coenzyme form.

In a study of these factors and their relationship to folic acid, crude liver digests were chromatographed on paper, and the positions of the active principles were determined on agar medium with *L. casei*. Folinic acid has an R_f value considerably greater than folic acid in butanol-ethanol-acetic acid solution; consequently, from the rate of migration the factors in liver replacing folic acid for *L. casei* could be determined. One of the two main bands in liver digest corresponded to folinic acid, but the other main band did not appear to be identical with folic acid but appeared to have a smaller R_f value than folic acid. Although these two main factors did not appear to be identical with folic acid, the absence of folic acid in liver could not be determined by this method, since higher concentrations of the other factors tend to mask the presence of small concentrations of factors migrating between the two main active bands.

In a series of synthesis experiments, folic acid has been converted to a factor which possesses activity analogous to folinic acid. When this method is applied to pteroyl-α-glutamylglutamic acid, two different bands of active principles are obtained by paper chromatography. One of these

corresponds to folinic acid and the synthetic material from folic acid. Similarly, three active principles are obtained from peteroyl-di-γ-glutamylglutamic acid, and one of these corresponds to the synthetic material from folic acid. Since the conditions of the synthesis are such that glutamyl radicals are cleaved to some extent, these active principles apparently are conjugates of glutamic acid, α-glutamylglutamic acid, γ-glutamylglutamic acid, and di-γ-glutamylglutamic acid with a modified pteroyl group.

Both folinic acid and the synthetic material from folic acid possess a single maximum of absorption of ultraviolet light at wave length 282 with an exceptionally high extinction coefficient. In very dilute acid, both the synthetic and natural material are destroyed with the formation of a substance which has the biological activity of folic acid but is not identical with folic acid.

Folinic acid appears to be approximately as effectively utilized as folic acid for all organisms and is considerably more active than folic acid for many organisms.

OPEN DISCUSSION

Dr. Buchanan: There is one point about which I have talked with Dr. Shive already, but I thought it should be mentioned. In the 4th reaction, that of the biosynthesis of thymine or thymidine, in which folic acid participates, I think it most probable that the 5-methyl group of thymidine is the single-carbon unit involved rather than the 2-carbon since this carbon appears to be derived from carbon dioxide rather than formic acid. At least, it appears that some carbon other than the carbon at position 2 is involved.

Dr. Shive: We have no data on which carbon of thymine is derived from the single-carbon units. All that our data indicate is that thymidine (or thymine) is an end product of a reaction catalyzed by a coenzyme derived from folic acid or p-aminobenzoic acid. We have obtained some evidence which is quite variable but which indicates the possibility of an

involvement of the tricarboxylic acids, e.g., *cis*-aconitic acid, in the biosynthesis of thymine.

Dr. Lampen: I might say that, in the work which we reported with the p-aminobenzoicless mutant of *Escherichia coli,* Dr. Jones and I found that homocysteine was active in replacing p-aminobenzoic acid. These results appeared to us to implicate a step previous to homocysteine in the action of p-aminobenzoic acid.

Dr. Shive tells me that a number of commercial samples of homocysteine (in our case a commercial sample was employed) are contaminated with considerable amounts of methionine. It may well be that this is the reason for the apparent discrepancy. We were somewhat perturbed about it at the time, and it may be that some methionine was not removed. We are checking that point.

I think that this is one apparent point of disagreement at the moment, but perhaps it is only due to contamination of homocysteine with methionine.

Dr. Shive: I would like to comment further on that subject. I do not want to indicate that the methylation step is the only involvement of the coenzyme derived from p-aminobenzoic acid in the biosynthesis of methionine. When sulfanilamide is present in the medium, *Escherichia coli* does not synthesize vitamin B_{12} in normal amounts. This effect of sulfanilamide on the vitamin B_{12} requirement could be related to the single-carbon unit in the 5,6-dimethylbenzimidazole part of the vitamin. Consequently, it is possible that a complete cycle is involved in which vitamin B_{12} functions in the formation of the coenzymes derived from p-aminobenzoic acid or the introduction of the single-carbon unit, and the final coenzyme in turn functions in vitamin B_{12} synthesis.

Thus, the effects of sulfanilamide would also include a deficiency of vitamin B_{12} which may be involved in other reactions such as the reduction of disulfide linkages to mercaptan groups. However, we have been unable to demonstrate this latter involvement under our testing conditions.

Dr. Price: I would like to mention something that might be of interest. There is, in the presence of folic acid deficiency, greatly diminished synthesis of purine, and there is also an accumulation of a compound which is apparently not the original labeled source of the single-carbon unit of the purine. At present, we have not characterized it, but presumably it is a precursor of inosinic acid. I would like to ask you to elaborate, Dr. Shive, on why you feel that folic acid, or a derivative, is involved in the closure of the imidazole as well as the aminoimidazolecarboxamide to form purines.

Dr. Shive: The only evidence which we have at present is the ability of aminoimidazolecarboxamide to prevent the toxicity of sulfanilamide, to some extent, for *Lactobacillus arabinosus*. Further evidence is offered by the observation that aminoimidazolecarboxamide does not accumulate in the medium of many organisms when the biosyntheses of purines are limited by deficiencies of the coenzyme derived from folic acid or p-aminobenzoic acid. Also, Dr. Snell of Wisconsin recently has shown that histidine exerts a "sparing action" on the purine requirement of *Lactobacillus casei* in the absence of folic acid. One probable point of this interrelationship is the single-carbon unit of both imidazole rings. While this evidence may not be conclusive, it is certainly suggestive of an involvement of folic acid or p-aminobenzoic acid in the formation of both single-carbon unit linkages in purines.

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MICROBIOLOGICAL SYSTEMS INVOLVING NUCLEIC ACID DERIVATIVES

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EIGHT FIGURES

The use of microbiological growth systems in studying biochemical reactions has become increasingly popular in recent years as the knowledge of nutritional requirements of various microorganisms has advanced. The sensitivity and reproducibility, as well as the ease with which microbiological growth systems can be handled, have made their use extremely practical in biochemical research. Many of the findings with microbiological systems have led to a clarification of the intermediary metabolism of higher species.

Recent studies (Snell et al., '48; Shive et al., '48a, b; Kitay et al., '49; Wright et al., '48; Skeggs et al., '48) with lactobacilli have demonstrated instances of relationship between their nutritive requirements and nucleic acid derivatives. Shive et al. ('48a) noted that *Lactobacillus lactis*, which requires vitamin B_{12} for growth (Shorb, '47), could utilize thymine desoxyriboside in place of vitamin B_{12}. Skeggs et al. ('48) reported that *Lactobacillus leichmannii* behaved in a like manner, responding either to vitamin B_{12} or thymidine. It was found that *Lactobacillus bifidus* (reclassified *acidophilus*) (Skeggs et al., '49) also was dependent on the presence of either vitamin B_{12} or thymidine in an otherwise complete semisynthetic medium. Figure 1 shows the response of the three organisms to vitamin B_{12}. *L. bifidus* requires in the order of 10 times as much vitamin B_{12} for growth as either

L. leichmannii or *L. lactis*. The ability of the three organisms to utilize thymidine in the absence of vitamin B_{12} for growth is illustrated in figure 2.

Kitay et al. ('49) subsequently showed that *L. leichmannii* can utilize the other desoxyribosides as well as thymidine. It has been tempting, therefore, to assume that the function of vitamin B_{12} is to catalyze the condensation of desoxyribose

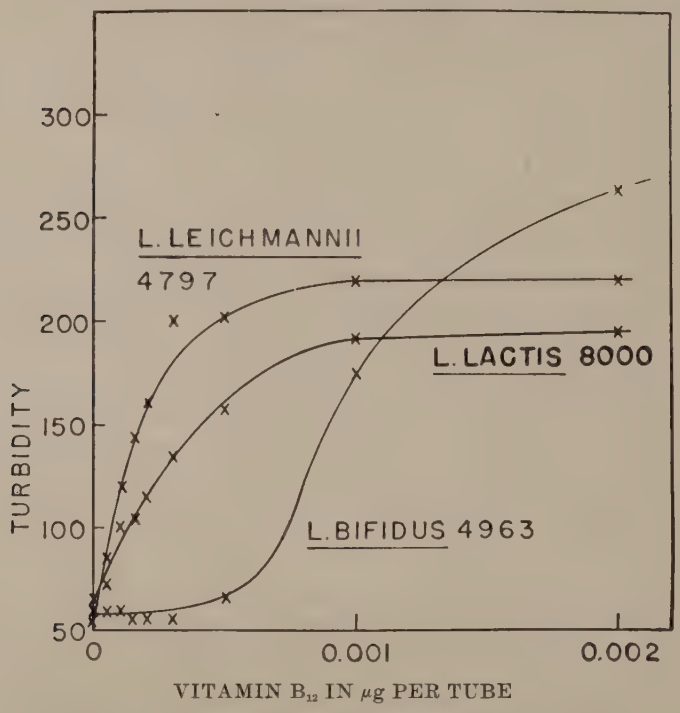

Fig. 1 Response of various lactobacilli to vitamin B_{12}.

with the purines and pyrimidines, and to assume that, in the absence of vitamin B_{12} or of the desoxyribosides, the organisms could utilize desoxyribonucleic acid (DNA). Figure 3 shows the responses of the three organisms to DNA. Only *L. bifidus* proved capable of attaining maximum growth in the presence of DNA. *L. leichmannii* grew poorly in the presence of DNA, and *L. lactis* failed to respond at all.

Our particular interests have been centered in the study of growth of *L. leichmannii* and *L. bifidus*. Let us first consider the behavior of *L. bifidus* when grown in the presence of DNA isolated from calf thymus (Skeggs et al., '50a). The organism utilizes DNA over a range of from 10 to 100 μg per 10 ml of basal medium. *L. bifidus* can utilize both highly polymerized and depolymerized preparations of DNA. While

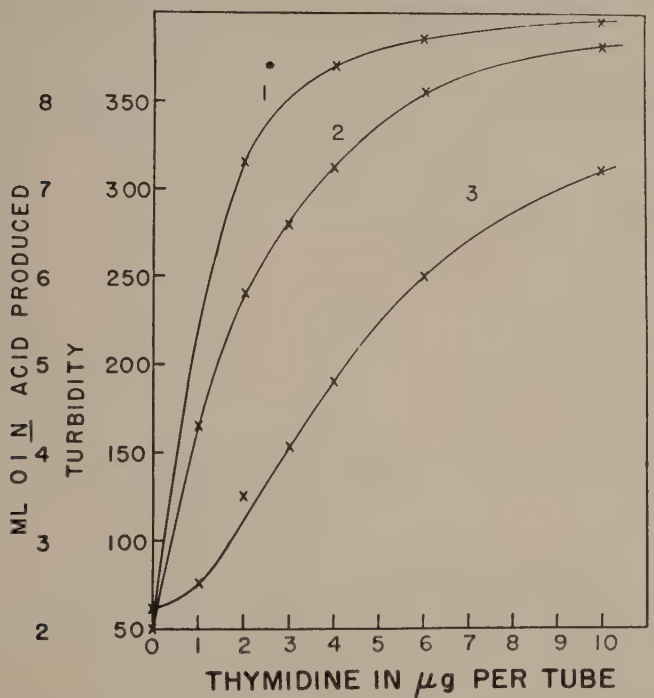

Fig. 2 Response of various lactobacilli to thymidine. 1. *L. lactis* 8000; 2. *L. bifidus* 4963; 3. *L. leichmannii* 4797.

some degradation of the polymerized molecule might be expected during autoclaving, the organism can utilize unheated preparations of DNA equally well. On agar plates, where the diffusion of the molecule is of importance in determining its activity, highly polymerized preparations are used less effectively than depolymerized preparations but in test tubes the degree of polymerization is not a factor of importance.

Bioautographic studies (Winsten and Eigen, '49) of various DNA preparations employed have given an R_f value of zero in butanol-acetic acid mixtures. Since the desoxyribonucleosides and presumably the nucleotides, have definite R_f values in such a mixture, it seems apparent that the organism utilizes intact DNA. (Furthermore, exposure of DNA to ultraviolet light produced equal losses in activity when measured by

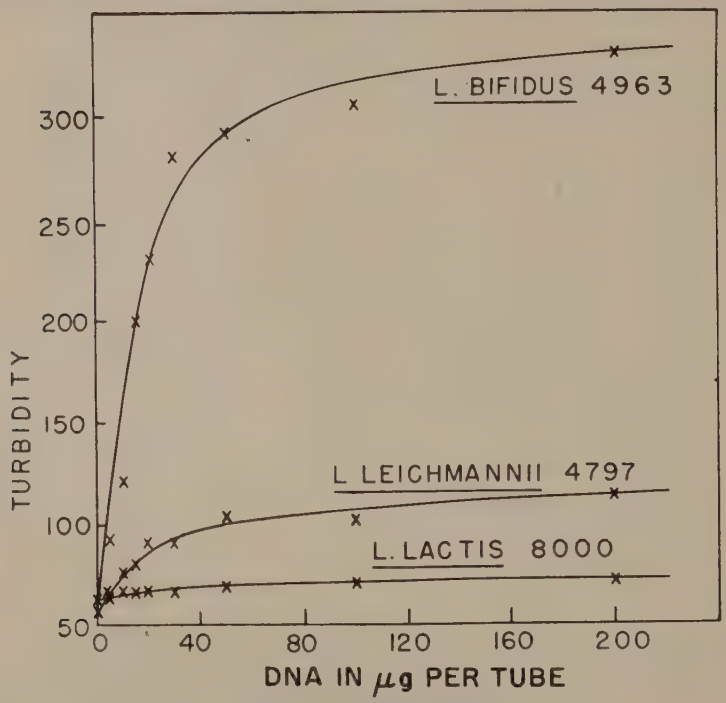

Fig. 3 Response of various lactobacilli to DNA.

either the cysteine reaction of Dische, '44, as modified by Stumpf, '47, or by microbiological assay.) Assay results on various samples of DNA compared favorably when duplicate runs were made with the chemical and microbiological methods.

The ability of *L. bifidus* to grow in response to DNA presumably would indicate the presence of desoxyribonuclease in the organism, but attempts to demonstrate the presence

of significant amounts of desoxyribonuclease (McCarty, '49) in either the cells or the supernatant have not, to date, been successful.

The ability of *L. bifidus* to utilize DNA for maximal growth suggested to us the possibility that the organism could be of value in searching for compounds that would inhibit the

TABLE 1

Inhibition studies with Lactobacillus bifidus

DNA μg/tube	COMPOUND μg/tube	ML 0.1 N ACID PRODUCED
0		1.40
5		3.10
10		4.05
15		5.60
20		9.40
30		11.55
50		12.75
	Propamidine	
10	10	5.45
10	100	1.25
10	1,000	1.10
10	10,000	1.90
	Na gentisate	
10	25	4.05
10	250	4.05
10	2,500	4.85
10	25,000	4.65
	Streptomycin	
10	10	2.85
10	100	1.20
10	1,000	1.20
10	10,000	1.35

utilization of DNA, on the basis that direct utilization of DNA might be analogous to the rapid turnover of DNA in tumor and regenerating cells (Furst et al., '50). Our procedures were therefore adapted so that a limiting amount of DNA was present in all tubes and plates, and the effect of various materials on the utilization of DNA could be ob-

served. The procedure employed is depicted in table 1. A constant amount of DNA (10 μg), which permitted partial growth of the organism, was placed in a series of tubes with an appropriate amount of water and basal medium and sterilized by autoclaving. Each compound to be tested was sterilized by passage through an ultrafine fritted glass filter, diluted and added aseptically in suitable amounts over a 1000-fold range. The test was seeded with *L. bifidus*, incubated at

TABLE 2

Effect of ribonucleic acid on utilization of desoxyribonucleic acid by Lactobacillus bifidus

RIBONUCLEIC ACID μg/tube	0.1 N ACID PRODUCED			INHIBITION RATIO RNA/DNA
	DNA 10μg/tube 72 hrs.	DNA 100 μg/tube 72 hrs.	DNA 1 mg/tube 72 hrs.	
0	3.20	9.70		
10	3.60	9.20		
20	3.60	9.30		
30	3.80	8.10		
40	3.00	9.05		
100	1.60	5.45		
200	1.00	6.60		ca. 10
300	1.00	4.35		
400	1.00	3.20		
1,000		2.00	10.50	
2,000		1.20	10.60	
3,000		1.10	7.10	
4,000		1.10	4.60	

Control — no DNA 1.35.

37°C. for three days and the resulting lactic acid titrated with 0.1 *N* sodium hydroxide. In this manner compounds that had no effect in inhibiting the growth of *L. bifidus* could be readily eliminated. Those which were active were then tested extensively to establish whether inhibition was due to blocking of DNA utilization. It was reasoned that if the inhibition was specific, the addition of greater amounts of DNA would increase the amount of compound required to inhibit the organism. Compounds such as streptomycin, nitroacridine,

protamine, and 8-hydroxyquinoline were found to be inhibitory, but inhibition of these compounds could not be reversed by the addition of greater amounts of DNA. Presumably, therefore, inhibition by such compounds was not due entirely to interference with DNA utilization.

The data in table 2 show that reversible inhibition was obtained with yeast ribonucleic acid (RNA). Highly purified according to the method of Elmore et al. ('48), RNA retained its ability to block the utilization of DNA. Degraded by

TABLE 3

Effect of adenylic acid on utilization of desoxyribonucleic acid by Lactobacillus bifidus

ADENYLIC ACID μg/tube	TURBIDITY					
	10 μg/tube DNA		100 μg/tube DNA		1 mg/tube DNA	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
0	105	155	145	329	230	364
10	97	125	135	294	216	363
20	85	142	120	278	214	362
30	81	98	106	272	206	355
40	75	87	103	243	210	361
100	67	81	80	270	250	364
200	66	81	70	216	165	290
300	66	78	77	206	155	189
400	61	81	72	164	147	153
1,000	61	86	64	121	140	123
2,000	62	84	66	110	124	128
3,000	61	80	64	106	138	131
4,000	61	77	66	109	114	124

Control — no DNA 24 hrs. — 75.
48 hrs. — 77.

alkaline hydrolysis, the inhibitory effect was exhibited by the degradation products. Nor was the inhibitory ability of RNA destroyed by autoclaving in the basal medium. Accordingly, the ribose nucleotides and nucleosides were investigated for their ability to replace RNA. Only the purine nucleotides, adenylic and guanylic acids, were capable of inhibiting the utilization of DNA. The pyrimidine nucleotides, the ribose nucleosides, and additional purines or pyrimidines were with-

out effect. Table 3 shows the effects obtained with yeast adenylic acid. This preparation of adenylic acid is a commercial preparation of unknown purity. Dr. Charles E. Carter ('50) kindly supplied us with two preparations of isomeric adenylic acids. The isomer identified as adenosine-3-phosphoric acid (adenlylic *b*) was found to be inhibitory. The isomeric adenylic acid *a* was essentially without effect in inhibiting the utilization of DNA. Dr. Henry Lardy provided

TABLE 4

Effect of guanylic acid on utilization of desoxyribonucleic acid by Lactobacillus bifidus

GUANYLIC ACID μg/tube	TURBIDITY					
	10 μg/tube DNA		100 μg/tube DNA		1 mg/tube DNA	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
0	105	155	145	329	230	364
10	96	127	130	305	230	375
20	98	126	136	299	226	365
30	83	141	128	294	230	362
40	78	86	114	291	234	360
100	66	89	90	290	224	362
200	69	89	73	287	195	329
300	69	90	73	277	181	281
400	68	84	73	249	160	237
1,000	68	92	78	154	150	141
2,000	64	80	78	111	165	145
3,000	65	78	78	113	172	173
4,000	62	77	73	88	191	177

Control — no DNA — 24 hrs. — 75.
48 hrs. — 77.

us with a highly purified preparation of adenosine-5-phosphoric acid which, while exhibiting inhibitory properties, was not as active as the adenosine-3-phosphoric acid.

Inhibition obtained with guanylic acid is typified by the data in table 4. Two commercial preparations of guanylic acid repeatedly have demonstrated this inhibitory ability. Neither adenylic acid, nor guanylic acid, nor a combination of the two exhibits the constant inhibition ratio obtained with RNA. Inhibition is much more pronounced during the

first 18 hours of growth in both cases, and prolonged incubation results in a gradual, irregular reversal. With RNA, inhibition can be demonstrated after 72 hours incubation. The prolongation of the inhibitory effect as observed with RNA may be due either to optimum proportions of the nucleotides in the intact nucleic acid or to a slow release of nucleo-

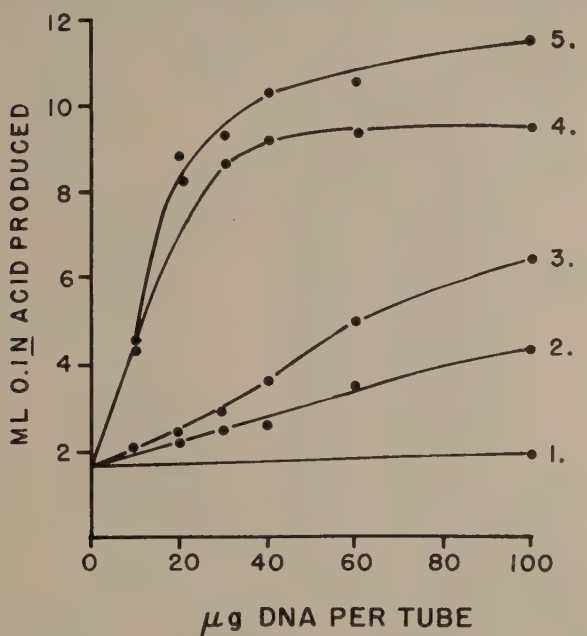

Fig. 4 Effect of purines and pyrimidines on utilization of DNA by *L. bifidus*. 1. No purines or pyrimidines; 2. Cytosine; 3. Uracil; 4. Uracil, adenine, guanine, xanthine; 5. Uracil, guanine, xanthine.

tides. Samples of RNA degraded by alkaline hydrolysis show some tendency to less prolonged inhibition than intact RNA samples, but are not as readily reversed as adenylic or guanylic acids.

The basal medium employed in these studies supplies 50 μg per tube of adenine, guanine, xanthine, and uracil. If the purines and uracil are omitted from the basal medium, *L. bifidus* cannot utilize DNA. Figure 4 shows that uracil, in

the absence of the purines, permitted partial growth. The purines, in the absence of uracil, did not support any growth. Neither cytosine nor thymine can replace uracil. Uridine, uridylic acid, and orotic acid can substitute for uracil in the nutrition of *L. bifidus*. Loring and Pierce ('44) found that a uracil-deficient strain of *Neurospora* was capable of utilizing cytidine and cytidylic acid, but not cytosine, in the absence of uracil. With *L. bifidus* a contradictory situation exists. If the regular amounts of adenine, guanine, and xanthine are included in the basal medium, the organism fails to utilize

TABLE 5

Effect of varying purines and pyrimidines on utilization of desoxyribonucleic acid by Lactobacillus bifidus

PRESENT IN MEDIUM 50 μg/tube [a]	GROWTH OF ORGANISM [b]	ADDED GUANYLIC ACID [c]	ADDED ADENYLIC ACID [c]
None	— to ±	S	O
A, G, X, U	++++	I	I
A, G, X	—	S	O
A, X, U	++++	I	I
A, G, U	++++	I	I
G, X, U	++++	I	I
U	++	S	I
A, G, X, T	—	S	O
T	—	S	O
A, G, X, C	—	S	O
C	±	S	O

[a] = A — Adenine; G — Guanine; X — Xanthine; U — Uracil; C — Cytosine; T — Thymine.

[b] = —, no growth; ±, slight growth; ++++, normal growth; ++, half maximum growth.

[c] = S, stimulation; I, inhibition; O, no effect.

cytidine or cytidylic acid. In the absence of any of the purines, however, a slight growth response to cytosine, cytidine, and cytidylic acid is obtained, which is not, however, equal to that seen with the uracil derivatives or orotic acid. The combination of uracil and any one of the purines permits utilization of DNA equal to that obtained when all 4 are present. The omission of adenine from the medium seems to permit slightly

better growth. It may be, therefore, that adenine competes with one of the other purines, since adenine and uracil together support better growth than uracil alone.

Under conditions where the lack of purines or uracil is limiting the ability of *L. bifidus* to grow in the presence of DNA, guanylic acid may be metabolized to some extent. Table 5 shows that when growth is so limited, guanylic acid

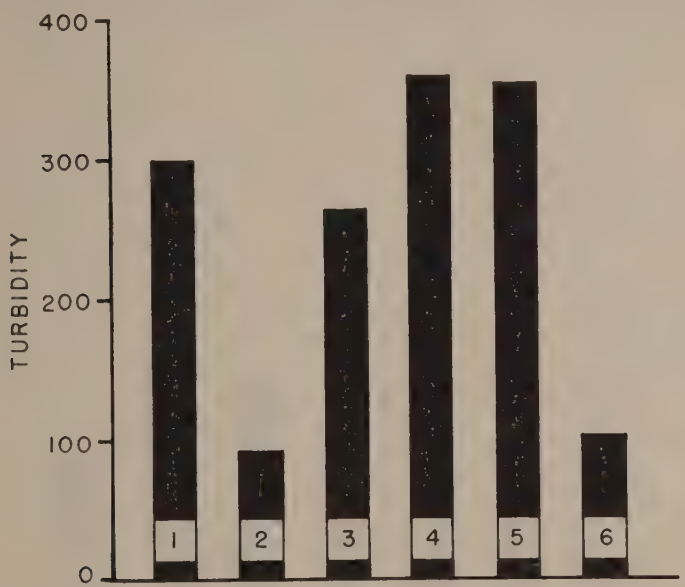

Fig. 5 Effect of purine nucleotides and ribonucleic acid on utilization of vitamin B_{12} (0.002 μg/tube) by *L. bifidus*. 1. No supplement; 2. 4 mg adenylic acid; 3. 4 mg guanylic acid; 4. 10 mg purified RNA; 5. 10 mg degraded RNA; 6. 10 mg crude RNA.

is stimulatory, but that when the combination of purines and uracil allows good growth to occur, guanylic acid is inhibitory. Adenylic acid does not exhibit this characteristic.

This leads us to a consideration of the fact that guanylic acid is not inhibitory when vitamin B_{12} replaces DNA in the nutrition of *L. bifidus*. Figure 5 shows that, in the presence of vitamin B_{12}, guanylic acid and RNA (purified and degraded) have very little effect on the growth of *L. bifidus*

after 24 hours incubation. Adenylic acid inhibits during early growth but when longer incubation is allowed, the inhibitory effects of adenylic acid disappear. The elimination of the purines and uracil from the medium prevent the growth of *L. bifidus* in the presence of vitamin B_{12}. The addition of uracil alone is not sufficient to permit growth to occur. Partial growth is obtained in the presence of uracil and one purine, either adenine, guanine, or xanthine, but maximum

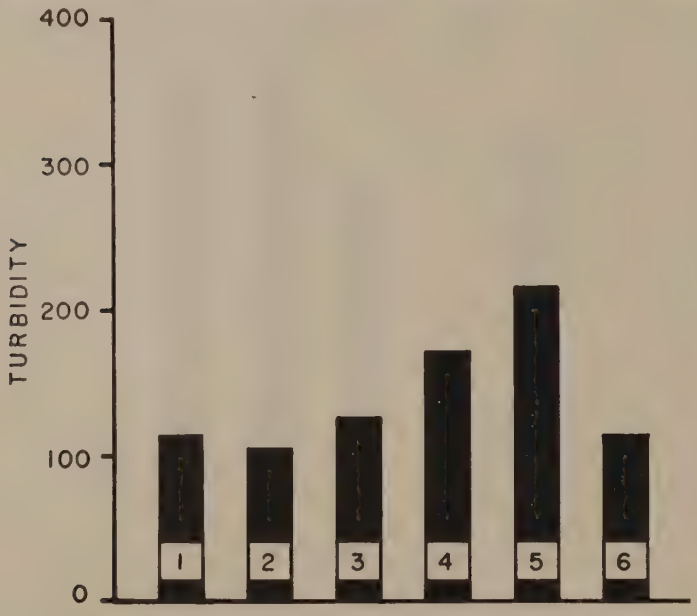

Fig. 6 Effect of purine nucleotides and ribonucleic acid on utilizaton of thymidine (2.0 μg/tube) by *L. bifidus*. 1. No supplement; 2. 4 mg adenylic acid; 3. 4 mg guanylic acid; 4. 10 mg purified RNA; 5. 10 mg degraded RNA; 6. 10 mg crude RNA.

utilization of vitamin B_{12} requires uracil and adenine plus either xanthine or guanine.

A few studies on the growth of *L. bifidus* in the presence of thymidine have been made. The scope of these studies has been limited by the fact that very little thymidine has been available. The effects of the purine nucleotides and RNA on the response of *L. bifidus* to thymidine are presented in figure

6. The nucleotide effects are much less apparent when thymidine is the limiting growth essential, and purified RNA is markedly stimulatory. It should be appreciated, however, that this work varies from that presented with DNA and vitamin B_{12} in that the amount of thymidine employed was limiting and did not permit maximal growth of the organism.

Figure 7 presents the data obtained when thymidine was employed in the growth of *L. leichmannii.* There is a great deal of similarity in the effect of the purine nucleotides and

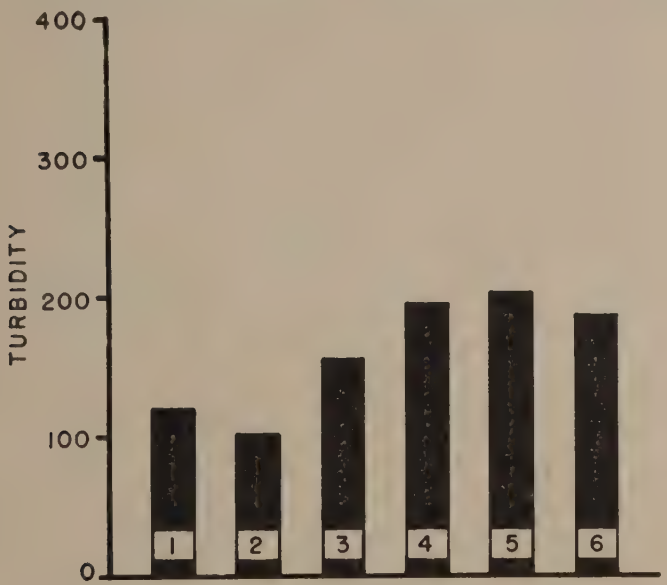

Fig. 7 Effect of purine nucleotides and ribonucleic acid on utilization of thymidine (2.0 μg/tube) by *L. leichmannii.* 1. No supplement; 2. 4 mg adenylic acid; 3. 4 mg guanylic acid; 4. 10 mg purified RNA; 5. 10 mg degraded RNA; 6. 10 mg crude RNA.

RNA to that obtained with *L. bifidus* and thymidine. Stimulation by guanylic acid and inhibition by adenylic acid are somewhat more apparent. The effects of the nucleotides and nucleic acids on the response of *L. leichmannii* to vitamin B_{12} are presented in figure 8 (Skeggs et al., '50a). Definite stimulation is apparent, particularly after 24 hours incu-

bation with RNA, uridylic and guanylic acids. Cytidylic acid may or may not be stimulatory. Adenylic acid (adenosine-3- or adenosine-5-phosphoric acid) is inhibitory during early growth but the inhibition disappears completely after 72 hours incubation. Neither the ribose nucleosides nor additional purines or pyrimidines exerts an effect in this growth system. Like *L. bifidus, L. leichmannii* does not grow in the absence of the purines and uracil. The inclusion of uracil,

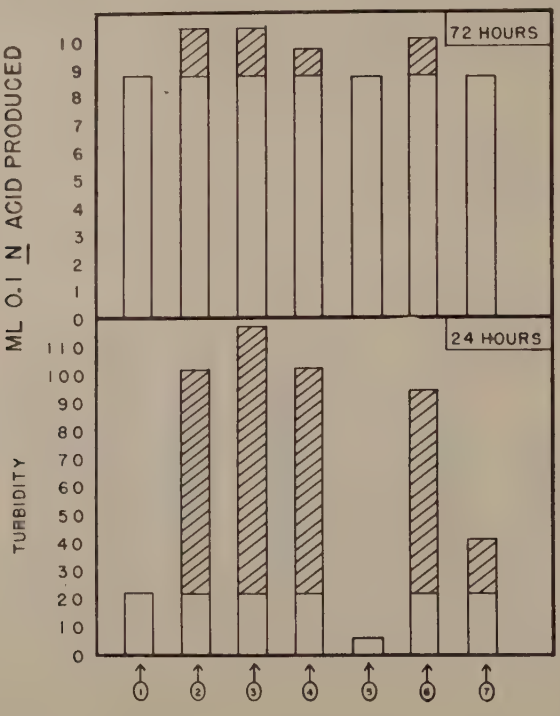

Fig. 8 Effect of nucleotides and nucleic acids on response of *L. leichmannii* to vitamin B_{12} (0.001 μg/tube). 1. No supplement; 2. 1.25 mg guanylic acid; 3. 1.25 mg uridylic acid; 4. 1.25 mg cytidylic acid; 5. 1.25 mg adenylic acid; 6. 1.25 mg RNA; 7. 1.25 mg DNA.

guanine, and xanthine is required for maximal growth to occur. In the absence either of uracil or of uracil and purines, both guanylic acid and to a lesser degree, adenylic acid, are able to promote partial growth.

Little attempt has been made to study the growth pattern of *L. leichmannii* with DNA. Growth of that organism in the presence of DNA is too limited.

At this point certain postulations may be attempted. Other investigators have presented evidence indicating that there is very little turnover of DNA in the mature cell, the ratio of RNA to DNA turnover (Furst et al., '50) being approximately 75 to 1. In regenerating cells, the turnover ratio of RNA to DNA is approximately 4 to 3. Geschwind and Li ('49) have shown that in fetal rat livers the ratio of RNA to DNA increases gradually from 0.98 to 1 to 2.8 to 1. The studies of Cunningham et al. ('50) indicate that the administration of a carcinogenic azo dye to rats increased the DNA content of liver cells while there was a decrease in cytoplasmic pentose nucleic acid. These data give more support to our theory that one of the functions of RNA in cellular metabolism is in the regulation of the turnover of DNA, and that the inhibition of DNA utilization by RNA in *L. bifidus* is universal to cellular metabolism. If this is so, this particular microbiological system could be a very useful tool in screening compounds that inhibit tumor growth. In this respect it is of interest to note that Barakan et al. ('48) found that adenylic and guanylic acids were of some value in retarding the growth rate of certain tumors in mice. We are, therefore, interested in testing compounds known to have some degree of activity in inhibiting tumor growth in order to determine whether this microbiological system might be of practical value. For this purpose, we have developed a disk plate assay procedure, in which a large number of compounds can readily be screened, and those showing definite zones of inhibition can be studied more exhaustively.

Considerable interest has been aroused in the triazolo compound, guanazolo, described by Kidder and Dewey ('49). Dr. Dewey supplied us with a generous sample of guanazolo, and the results of our screening procedures are shown in table 6. The basal medium supplied 50 μg per tube of guanine, and the amounts of guanazolo were greatly in excess of those

required to inhibit *Tetrahymena geleii*. Marked inhibition was obtained only in the growth system employing vitamin B_{12} and *L. bifidus*. The reversal of this inhibition by increased amounts of guanine has not been attempted, as yet.

Regardless of the validity of our reasoning concerning the application of these microbiological systems in the screening of compounds that inhibit tumor growth, the conclusion seems justified that the metabolic pathways utilized by these organ-

TABLE 6

Effect of guanazolo in microbiological systems

GUANAZOLO μg/tube	LACTOBACILLUS BIFIDUS				LACTOBACILLUS LEICHMANNII	
	DNA/tube			B_{12}/tube	B_{12}/tube	
	10 μg	100 μg	1 mg	0.01 μg	0.0005 μg	0.005 μg
0	3.30	9.20	11.05	12.55	5.70	6.35
100	4.30	8.15	8.25	10.20	5.60	5.70
200	3.35	7.35	8.30	9.15	5.80	5.90
300	4.95	7.55	8.70	8.40	5.80	5.75
400	3.15	7.15	8.70	8.60	6.05	6.20
1,000	4.45	6.25	8.65	6.10	6.15	6.70
2,000	2.25	5.10	7.65	2.60	5.20	5.30
3,000	2.30	3.45	6.75	2.00	4.80	5.00
4,000	2.15	3.70	5.50	1.95	4.20	4.40
Guanylic acid						
4,000	2.0	5.40	5.50	13.70	8.90	8.15
Adenylic acid						
4,000	1.7	3.55	6.90	14.05	6.30	7.10

isms are dependent in part upon the available nutrients. Therefore, by regulating the composition of the basal medium, frequently one can examine the factors that affect a given metabolic system. The advantages of using microbiological systems are at once obvious since they combine a means of chemical analysis with a biological system, wherein results can be obtained in from 24–72 hours. An excellent illustration of differential analyses possible with microbiological systems

recently was described by Hitchings and co-workers ('50). When thymine supplied the necessary growth requirements of *L. casei,* 5-bromouracil specifically blocked the utilization of thymine. 4-Amino folic acid, which one would expect to be a specific folic acid antagonist, on the other hand inhibited the utilization of folic acid but had very little effect on the utilization of thymine.

TABLE 7

Summary of effects of purine nucleotides and RNA on growth of Lactobacillus bifidus and Lactobacillus leichmannii [a]

PRESENT IN MEDIUM	EFFECT OF		
	RNA	Guanylic acid	Adenylic acid
DNA	I	I	I
Vitamin B_{12}	S	S	I
Thymidine	S	S	I

[a] — S = Stimulation; I = Inhibition.

Table 7 represents an attempt to condense and summarize the data. The effects of the purine nucleotides and RNA on the growth systems are represented either as inhibitory or stimulatory without regard to the degree or duration of the effect.

In summary, we have presented evidence that nucleic acid derivatives exhibit regulatory mechanisms in two microbiological growth systems. It is hoped that these systems can be adapted to studies of cellular metabolism.

OPEN DISCUSSION

Chairman Foster: Have you any explanation for the ability of guanylic acid to support partial growth in the absence of the purines and pyrimidines?

Mrs. Skeggs: We have no explanation.

Dr. Schmidt: When you add ribonucleic acid to the medium, is there any evidence that enzymes are excreted that first transform the ribonucleic acid into lower metabolites?

Mrs. Skeggs: We have no evidence on that point.

DR. LAMPEN: Have you gone into the question of how much of the activity of DNA is really the result of the intact DNA molecule? Can thymidine, for example, completely replace DNA?

MRS. SKEGGS: Thymidine will completely replace DNA. We assume that DNA is depolymerized and split by the organism in the process of assimilation, but we have been unable to demonstrate the presence of a depolymerase.

DR. LORING: We have some results on the utilization of cytidylic acid by pyrimidine-deficient *Neurospora* that are somewhat comparable to those showing differences in inhibitory activity of adenylic acids *a* and *b*. Originally, in studying the utilization of cytidylic acid by this strain we obtained a fairly high growth curve. That preparation of cytidylic acid was prepared enzymically by ribonuclease action. When we used cytidylic acid prepared chemically we found, surprisingly, that we were getting a much lower growth curve. The difference in the two preparations was never explained until now perhaps, with the isolation of isomeric cytidylic acids. It may be that they will differ in the extent to which they are utilized by pyrimidine-deficient *Neurospora*.

DR. FOSTER: Have you any information about compounds of this type on bacteria other than lactobacilli?

DR. SPIZIZEN: We have performed some experiments on the growth of bacteriophage T_2 in *Escherichia coli*, in which we have observed a short time inhibition with adenylic acid of the same type that is shown here. In addition to that, guanylic acid has a highly stimulatory effect, in a highly synthetic medium.

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